3

Circulating Markers in Gastroenteropancreatic Neuroendocrine Tumors (GEP NETs)

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1. Introduction

Neuroendocrine Tumors (NETs) constitute a heterogeneous group of neoplasms which originate from neuroendocrine cells of diffuse endocrine system. They may synthesize, store, and secrete peptides and neuroamines that can cause distinct clinical syndromes. On the other hand, many are clinically silent until late presentation with mass effects (1).

Gastro-Entero-Pancreatic (GEP) NETs originate from both pancreatic islet cells or gastroenteric tissue (from diffuse neuroendocrine cells distributed throughout the gut) and are rare neoplasms, representing about 2% of all the gastrointestinal tumors. Due to their rarity, they are difficult to diagnose and the begnning of the diagnostic process is often based on the measurement of circulating markers, before planning expensive and invasive diagnostic tests (2, 3). A critical point is that the frequent late diagnosis of NETs is due to failure to identify symptoms or to establish the biochemical diagnosis; in fact 60-80% of NETs are metastatic at diagnosis. A prompt identification by the use of specific biomarkers is therefore useful to recognize these tumors (1).

Circulating tumor biomarkers can be divided into general and specific biomarkers. The neuroendocrine cells that give rise to NETs have many common features, including the synthesis of peptides, biologically inactive, that act as general markers, but have also the capacity to secrete a variety of specific biomarkers that characterize a precise biochemical function (4). Individual amines and peptide hormones are indeed specific to certain types of NETs (**Table 1**).

2. General biomarkers

There are several families of secretory proteins found in high concentrations in neuroendocrine cells and, in particular, neuroendocrine tumor cells.

They include the granins, neuron specific enolase (NSE), and pancreatic polypeptide (PP). Both chromogranin A (CgA) and NSE show increased concentration levels in many patients with NETs, but CgA is recognized as the most effective and the only general biomarker that has been extensively investigated (1-5).

Tumor Site	**Syndrome**	**Symptoms**	**Biomarkers**
Gastric (type 1 and 2)	None	Upper GI	CgA, gastrin
Gastric (type 3)	None	Upper GI	CgA
Duodenal	Zollinger-Ellison	Epigastric pain, peptic ulcer, diarrhea, GERD	CgA, gastrin (>50%), PP (35%), Somatostatin (<10%)
Ileal	Carcinoid	Diarrhea, flushing, sweating	CgA, serotonin, NKA and SP, 5HIAA
Appendix	Carcinoid	Diarrhea, flushing, sweating	CgA, serotonin, HIAA, NKA
Rectal	None	None	CgA, PYY(10%)
Meckel diverticulum	Zollinger-Ellison	Epigastric pain, peptic ulcer, diarrhea	CgA, gastrin (>50%)
Pancreas			
Insulinoma	Whipple's triad	Hypoglycemia, dizziness, sweating	CgA, insulin, pro-insulin, C-peptide
Gastrinoma	Zollinger-Ellison	Epigastric pain, peptic ulcer, diarrhea	CgA, gastrin, PP (35%)
VIP-oma	WDHA	Watery diarrhea	CgA, VIP
Glucagonoma	None	Necrolytic migratory erythema	CgA, glucagon, glycentin
Somatostatinoma	None	Mild diabetes, gallstones	CgA, somatostatin
PP-oma	None	None	CgA, PP
Non functioning	None	None	CgA, PP

CgA=Chromogranin A; 5-HIAA= 5-hydroxyindoleacetic acid; PP= Pancreatic polypeptide; VIP= vasointestinal peptide; NKA= neurokinin A; PYY= peptide YY

Table 1. Syndromes, symptoms and secretory products from GEP NETs

2.1 Granins

The chromogranin family consists of at least three different water soluble acidic glycoproteins (CgA, CgB, and secretogranin II, sometimes called chromogranin C). These proteins are 27 to 100 kDa in size and contain 10% acidic (glutamic or aspartic acid) residues, as well as multiple single and dibasic amino acid residues. All of the granins are found as major components of the soluble core of dense-core secretory granules in NE cells and are secreted from these cells in a physiologically regulated manner. Granins are major constituents of large dense-core secretory vesicles and are co-secreted with peptide hormones and amines. Electron dense or translucent secretory granules are in fact prototypical features of the neuroendocrine cells (1-6).

2.1.1 Chromogranin A (CgA)

Chromogranin A (CgA) has been claimed to be the best general neuroendocrine marker so far available. CgA is a 49 kDa monomeric, hydrophilic, acidic glycoprotein of 460 amino acid and is widely expressed in neuroendocrine cells, where it constitutes one of the most abundant components of secretory granules, and it is secreted from neuroendocrine-derived tumors including functioning and non-functioning GEP NETs, pheochromocytomas, neuroblastomas, medullary thyroid carcinomas and some pituitary tumors. CgA is secreted to the extracellular space, so it's easily detectable in the blood. CgA is co-secreted with the amines and peptides that are present in the neurosecretory granules even if it can be elevated in both functionally active and non-functional NETs. CgA seems to be a "common denominator" peptide in all the components of the diffuse neuroendocrine system (7).

The precise function of CgA remains unknown, but it is thought to be involved in the packaging and processing of neuropeptide precursor and peptide hormones. It may also play a role in the organization of the secretory granule matrix. Moreover CgA has diverse physiological interactions: CgA (or its derivatives) is an inhibitor of catecholamine, insulin, and leptin, having a role in carbohydrate and lipid metabolism; moreover it inhibits parathormone secretion; on the other hand, CgA increases glucagon and amylase release. In addition to its effects on endocrine organs, CgA also regulates reproductive functions and has a role also in the regulation of cardiovascular function: CgA elevations have been reported in essential hypertension (CgA levels correlates with the severity of hypertension) and in chronic heart failure correlating with grade of cardiac dysfunction and mortality. A role of CgA in the regulation of inflammatory response has also been described. In fact increased CgA levels correlate with serum TNF-α receptor levels in a number of inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, chronic obstructive pulmonary disease and chronic heart failure. Patients with sepsis show the highest increase of CgA; CgA positively correlates with inflammatory markers as C-reactive protein and procalcitonin. It remains to be elucidated the pathophysiological relevance of these correlations. Some authors suggest that CgA participates in a negative feedback that limits the activation of endothelial cells (1).

Circulating CgA concentrations are sensitive even if non specific markers of NETs. CgA has been reported to be more sensitive than urinary 5-hydroxyindoleacetic acid (5-HIAA) as well as than pancreatic polypeptide concentrations. The highest values were noted in ileal NETs (200 times the upper normal limit) and IN GEP-NETs associated with MEN1 (150 times the upper normal limit) while gastric type I, pituitary, and parathyroid tumors had lower values (ranging from 2 to 4 times normal). Both functioning and nonfunctioning pancreatic NETs had intermediate values (60–80 times the upper limit of normal) as did Zollinger- Ellison Syndrome (ZES), multiple endocrine neoplasia (MEN)-1, type II and III gastric entero-chromaffin-like (ECL)omas (80–100 times normal). It has also been proposed that CgA is more frequently elevated in well-differentiated tumors compared to poorly differentiated tumors of the midgut, suggesting that the loss of CgA expression in poorly differentiated neuroendocrine carcinomas indicates their incomplete or partial endocrine differentiation. In fact, poorly differentiated NE carcinomas rarely express CgA because of the rarity of large, dense-core granules. The presence of high plasma levels of CgA at diagnosis is an independent prognostic factor that indicates a reduced overall survival.

Effective treatment is often associated with decrease in CgA values. CgA correlates with tumor burden and recurrence. Measurement of CgA may help in the effective diagnosis of NET and has a major utility in predicting disease recurrence, outcome, and efficacy of therapy, so delineating the prognosis (5).

Elevated CgA can occur in normal individuals and in patients with non-NET tumors, although the levels are usually lower than in patients with NET. Less than 1% of CgA tests that are more than 20 times greater than the reference range are false positive. Levels of CgA secretion vary on a day-to-day basis in healthy subjects as well as in individuals with NETs. The mean day-to-day variation of CgA is approximately 25%. Food intake may increase CgA levels, therefore, CgA should be measured in fasting patients to ensure standardization of the results. There are conflicting results on the impact of exercise on CgA. Significant increases in CgA concentration have been reported in healthy subjects, but in patients with heart disease, long-term exercise had no impact on CgA. Finally extreme physical stress also causes CgA elevations. High-serum levels of CgA have also been demonstrated in patients with other malignancies including colon, lung, breast, liver and prostate cancer. Overall CgA has been found to be clinically informative and moderately sensitive in the majority of the studies, and more sensitive than NSE. In prostate cancer elevated CgA seems to indicate a poor prognosis; in small-cell lung carcinoma CgA levels were more frequently elevated and were also higher in cases of more extensive disease; NE differentiation occurs in 34% of primary colorectal cancer (1).

False-positive elevation of CgA may also occur in the following non-neoplastic circumstances: impaired renal function, Parkinson disease, untreated hypertension and pregnancy, steroid treatment or glucocorticoid excess, chronic atrophic gastritis (type A), treatment with proton pump inhibitors (PPI), inflammatory bowel disease, liver disease, hyperthyroidism. In renal failure CgA increases due to a decreased plasma clearance, reaching levels found in neuroendocrine neoplasia. In autoimmune chronic atrophic gastritis, elevated circulating CgA levels are caused by chronic hypergastrinemia and stimulation of ECL cell proliferation. Raised circulating CgA levels in addition to raised gastrin in atrophic gastritis, confounds the diagnosis of gastrinoma in many patients who present with dyspeptic symptoms. But the major cause of elevated CgA levels is the widespread use of PPIs and other acid suppressive medications. All PPI users, even with low dosage (10 mg/d) have elevated fasting CgA levels. The normalization of CgA levels occurs by withdrawal of PPI in 1-2 weeks (1, 10).

There is no universal standard calibration for serum or plasma chromogranin A assays. In addition, different chromogranin A assays, which use different antibodies or antibody combinations, will display different cross-reactivity with the various chromogranin A fragments. Therefore, reference intervals and individual patient results differ significantly between different chromogranin A assays and cannot be directly compared. Several commercially available radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) have been developed for the measurement of circulating CgA concentrations. Moreover many diagnostic laboratories use in-house assays. The three main different commercial kits are CgA-RIA CT (CIS Bio International, Gif-sur-Yvette Cedex, France), Dako CgA ELISA kit (Dako A/S, Glostrup, Denmark), and CgA EuroDiagnostica (ED) (Malmo¨, Sweden). All three assays use different standards. In the CIS kit, CgA concentration is expressed in ng/ml and normal range is < 99μg/l, while with the

Dako assay results are expressed in U/l, and normal range is within 19 U/l; with the ED kit CgA levels are expressed in nmol/l and normal range is < 41 nmol/l. Concerning plasma and serum measurement, a strong positive linear relationship has been reported between plasma and serum CgA values, indicating that CgA measurement can be undertaken in both sample types. In conclusion, because CgA concentrations are of considerable clinical relevance, substantial characterization and standardization to ensure uniform reporting are needed (1, 7, 8).

2.1.2 Other granin family peptides

The granin family comprises eight members including CgA and its derivated peptides, CgB, CgC (secretogranin II [SgII]), SgIII, SgIV, SgV, SgVI and VGF, but their value as circulating markers for endocrine tumors has not been investigated extensively extensively (1).

Several other CgA-derived peptides, resulting by posttranslational processing have been isolated from extracts of human endocrine tumors. These molecules results in a series of smaller biologically active peptides, such as pancreastatin (corresponding to CgA residues 250–301), catestatin (corresponding to CgA residues 352–372), and vasostatin I and II (corresponding to CgA residues 1–76 and 1–113, respectively). These CgA derived peptides affect secretion of other hormones, play a role in vasoconstriction, and regulate metabolism. Among them, the most clinically interesting is pancreastatin. An endoprotease, the prohormone convertase-1 (PC-1), is involved in the processing of the precursor protein chromogranin A (CGA) to a smaller peptide called pancreastatin (PST), a 49-aminoacid peptide that inhibits insulin secretion, somatostatin release, exocrine pancreatic secretion and gastric acid secretion. PST is found in human stomach- and colon extracts and in a liver metastasis of gastrinoma. Pancreastatin was used before the complete sequence of CgA had been elucidated and before there were any reliable assays that could measure the whole molecule of CgA, as an epitope for antibody production. Pancreastatin antisera were used in immunohistochemistry and RIA to assess the presence of CgA in cells and the concentration of CgA in the circulation. But pancreastatin levels do not equale to CgA concentrations in the circulation. The molecule was found to be significantly increased in patients with NETs metastasized to the liver and concentrations are proportional to the number of hepatic metastases. Monitoring of liver metastases may remain the main advantage of pancreastatin assay (2). It is interesting that pancreastatin is not increased in patients with gastric achlorhydria or hypochlorhydria. Thus, false-positives are less problematic with the pancreastatin assay. It may be a very early biomarker for liver tumor activity, even when CgA is normal (5).

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. Unlike CgA, CgB does not seem to have increased concentrations in patients with renal failure, in patients with atrophic gastritis, or those receiving acid-suppressing therapy. The interest to measure CgB in addition to CgA in patients with GEP NETs is therefore increased. Moreover, in tumors where CgA is not found, CgB may be increased. Such patients include those with MEN 1 and those with tumors in the duodenum or rectum. In addition, CgB is a major granin of the human adrenal medulla and may be a more sensitive marker of pheochromocytomas (2, 5).

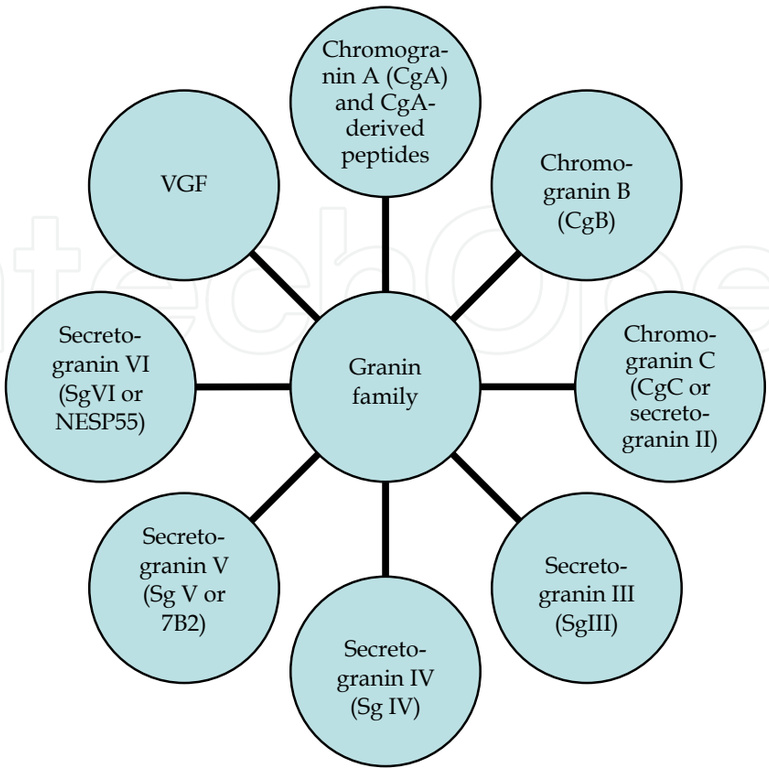

Fig. 1. The granin family and fragments of CgA. The 8 granin proteins include chromogranin A, CgB, CgC (SgII), SgIII, SgIV, 7B2 (SgV), neuroendocrine secretory protein 55 (NESP55 or SgVI), and VGF nerve growth factor–inducible (VGF).

2.2 Pancreatic polypeptide (PP)

Circulating 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), it also has effects on hepatic glycogen levels and gastrointestinal secretions.

Elevated PP concentration are found in patients with NETs, both pancreatic (20-50%) and gastrointestinal carcinoids (30-50%). Before methods for the measurements of CgA were available, PP was used as a general marker for endocrine tumors, although it is poorly specific. PP is now useful in the diagnosis and monitoring of NETs where no other general marker is raised and in PP-omas. High levels of circulating PP can also be found in diabetes, renal impairment, chronic inflammation, alcoholism and in elder patients. Its secretion is increased after a protein meal, fasting, exercise, and acute hypoglycemia and is decreased by somatostatin and intravenous glucose (2, 11).

2.3 Neuron Specific Enolase (NSE)

NSE is the neuron-specific isomer of the glycolytic enzyme 2-phospho-D-glycerate hydroxylase or enolase. This isomer is present in neurons and neuroendocrine cells and can be used as a biomarker for tumors derived from these cells. As well as CgA, NSE is a marker

useful for the diagnosis and the monitoring of patients with neuroendocrine tumors (especially neuroblastoma, small cell lung cancer, less important for GEP NETs); it has other several applications, including the assessment of neuronal damage during stroke. Elevated NSE levels are indicative of poorly differentiated tumors. NSE levels seems not to be related to any secretory activity of the tumor (11, 12).

3. Specific biomarkers

In addition to general markers, there are biomarkers specific for particular GEP-NET associated syndromes. The most typical is carcinoid syndrome and the specific marker is 5-Hydroxyindole Acetic Acid. Other specific markers including insulin, gastrin, vasoactive intestinal peptide, glucagon, bradykinin, substance P, neurotensin, human chorionic gonadotropin, neuropeptide K, and neuropeptide L are each of some value in precisely defining the functionality of individual NETs (see above **Table 1**).

3.1 5-Hydroxyindole acetic acid (5-HIAA)

5-HIAA is the urinary breakdown of serotonin, which is synthesized and stored in enterochromaffin cells of the gastrointestinal tract (80% of total body serotonin content), in dense granules of platelets and in the serotoninergic neurons of the central nervous system. Serotonin is a ubiquitous tryptophan-derived biogenic amine, involved in homeostasis, vasoconstriction and neurotransmission (7).

Carcinoid syndrome is the typical clinical picture of metastatic ileal carcinoid, occurring in about 18% of patients and is characterized by flushing, diarrhea, abdominal pain; less frequent events are lacrymation, profuse sweating, telangiectasias, cardiac fibrosis, and cutaneous manifestations pellagra-like due to lack of niacin. This syndrome is caused by the massive release of serotonin, which is no longer metabolized in the liver, and other substances, such as tachykinins, prostaglandins, and bradykinins (3) (**Table 2**).

Clinical features	**(%)**	**Characteristics**	**Mediators**
Flushing	90	*Foregut* tumors: prolonged fit, red-purple, localized to face and trunk. *Midgut* tumors: quick fit, pink-red.	Serotonin, histamine, P substance, prostaglandins
Diarrhea	70	Secretory	Serotonin, histamine, VIP, prostaglandins, gastrin
Abdominal pain	40	Long lasting	Obstruction, hepatomegaly, intestinal ischemia, fibrosis
Profuse sweating	15		Serotonin, histamine
Telangiectasias	25	Face	Unknown cause
Heart disease	30 (right) 10 (left)	Valvulopathies (tricuspid valve, pulmonary valve). Right heart failure. Dyspnea	P substance, serotonin
Pellagra	5	Dermatitis	deficit of niacin

Table 2. Characteristics of carcinoid syndrome

This syndrome is typical of metastatic well-differentiated midgut NETs, even if other clinical conditions may mimic symptoms and signs (**Table 3**).

Clinical Condition	**Tests**
Carcinoid	CgA, Serotonin, 5-HIAA, PP, VIP
VIP-oma	CgA, PP, VIP
Medullary carcinoma of the thyroid	CgA, CEA, Calcitonin, Ca++ infusion, RET proto-oncogene
Pheocromocytoma	CgA, Plasma free metanephrines, urine metanephrines, VMA, Epi, Norepi, glucagon stimulation, MIBG
Diabetic autonomic neuropathy	HRV, 2hs PP glucose
Menopause	FSH
Epilepsy	EEG
Panic attack	Pentagastrin, ACTH
Mastocytosis	Plasma histamine, urine tryptase
Mitral valve prolapse	Cardiac echo

CgA=Chromogranin A; 5-HIAA= 5-hydroxyindoleacetic acid; PP= Pancreatic polypeptide; VIP= vasointestinal peptide; CEA= carcino-embryonic antigen; VMA= vanillylmandelic acid; Epi= epinephrine; Norepi= norepinephrine; MIBG= metaiodobenzylguanidine; HRV= heart rate variability; 2hs PP= 2-hour postprandial blood sugar; FSH= follicle-stimulating hormone; EEG= electroencephalography; ACTH= adrenocorticotropic hormone.

Table 3. Differential diagnosis of flushing and diagnostic tests

The 24-h measurement of 5-HIAA is a useful specific marker for serotonin-producing NETs. The overall sensitivity and specificity of urinary 5-HIAA in the presence of the carcinoid syndrome is 70 and 90%, respectively. Therefore this marker is the most frequently performed assay in the clinical setting of the carcinoid syndrome. Midgut carcinoids are most liable to produce the carcinoid syndrome with 5-HIAA elevation, thus attesting to a high specificity in this setting (approximately 75% of midgut NETs are associated with a positive urinary 5-HIAA test). Functional symptoms in NETs originating from the midgut are in fact mostly due to the secretion of 5-hydroxytryptophan (5-HTP) or serotonin. The sensitivity is lower in patients with midgut carcinoid tumors without the carcinoid syndrome and in patients with fore- and hindgut NETs due to less serotonin production from these tumors than midgut ones. Elevated 5-HIAA levels in the urine are highly suggestive of an ileal NET, although some NETs found in the lung and pancreas also secrete serotonin (7, 11).

High-performance-liquid-chromatography (HPLC) with electrochemical detection is currently recommended to measure 5-HIAA. In some laboratory automated assays or those using mass spectrometry are available.

There are false positive 5-HIAA urinary levels as well as false negative ones. Some foods contain high levels of serotonin which may increase the levels of urinary 5-HIAA and their consumption should be avoided 3 days prior to urine collection (i.e. plums, pineapples, bananas, eggplants, tomatoes, avocados, and walnuts). For this reason patients need to be on a diet free of tryptophan/serotonin-rich foods to avoid false elevations in urinary 5-

HIAA. Untreated patients with malabsorption (celiac disease, tropical sprue, Whipple disease, intestinal stasis and cystic fibrosis), may have increased tryptophan metabolites. Also certain medications may interfere with the assay: paracetamol, fluorouracil, methysergide, naproxen and caffeine may cause false positive results. On the contrary levodopa, aspirin, adrenocorticotrophic hormone (ACTH), methyldopa, and phenothiazines may give a false negative results (13). Somatostatin analogs are known to decrease levels of 5-HIAA. Moreover, patients with renal impairment and those with hemodyalisis may have falsely low 5-HIAA levels.

5-HIAA does not seem to be a useful prognostic factor in patients with carcinoid syndrome, because of the fluctuating release of serotonin in NETs of the midgut. On the other hand, several studies found high 5-HIAA levels to be an independent survival factor. Overall, in these studies, higher concentrations of urinary 5-HIAA are associated with a worse prognosis, and persistently low 5-HIAA excretion predicts more favorable survival in patients with disseminated disease. The intra-individual variation of 5-HIAA may be high. When the collection is required for the diagnosis it is useful to have two consecutive 24-hours collections and to take the mean value (7).

Serotonin plays a key role in development of peritoneal and cardiac fibrosis via activation of the 5HT2B receptor and a cascade of connective tissue growth factors. Reductions in plasma serotonin levels correlate with a decreased incidence of carcinoid heart disease (CHD). Moreover, urinary 5-HIAA excretion also correlates with the severity of CHD and prognosis in patients with carcinoid syndrome.

3.2 Insulin

Insulin is a peptide hormone composed of 51 amino acids and has a molecular weight of 5808 Da. It is produced in the islets of Langerhans in the pancreas, within the β-cells. In β-cells, insulin is synthesized from the proinsulin precursor molecule. Insulin is a hormone central in the regulation of carbohydrate and fat metabolism in the body. Since the main action of insulin is reducing blood glucose levels, by increasing glycogen synthesis and promoting storage of glucose in liver (and muscle) cells, insulin excess (such as an insulinoma) induces hypoglycemia. In patients with suspected insulinoma, the insulin and its precursors or breakdown products should be tested, even if further biochemical tests include the 72-hour fast, which is the gold standard for establishing the diagnosis of insulinoma. Insulinomas secrete proinsulin, insulin and C-peptide intermittently, and, although insulin concentrations in the circulation may often be within reference range, insulin is at most times inappropriately high for the blood glucose concentration (14).

Insulinoma is the most common secretory NET of the pancreas that produces a symptomatic clinical syndrome. More than 80% of insulinomas are benign. Insulinoma is uncommon, although it is the second most common pancreatic NET to occur in patients with MEN1 (5).

3.3 Gastrin

Gastrin is a hormone that stimulates secretion of gastric acid (HCl) by the parietal cells of the stomach and aids in gastric motility. It is released by G cells in the stomach, duodenum, and the pancreas. Gastrinoma (gastrin-producing tumor) is the second most common

secretory pancreatic NET. It can rise from both cells in the duodenum and in the pancreas, with just more than half malignant at presentation. Approximately 25% to 35% of gastrinomas are associated with MEN1. Gastrinoma is the most common GEP NET associated with MEN1. Gastrinomas secrete gastrin but gastrin can circulate in numerous forms. Progastrin, gastrin 34, gastrin 17, and C-terminally extended gastrins may all circulate in high concentrations in patients with gastrinoma. In the Zollinger-Ellison syndrome, gastrin is produced at excessive levels. Normal values are generally less than 100 pg/mL (2, 5).

Gastrinoma is not the only cause of hypergastrinemia, since there are several causes for hypergastrinemia that often require numerous and expensive diagnostic investigations. Hypergastrinemia is most frequently due to hypochlorhydria and only seldom the underlying cause is gastrinoma. The most frequent condition that causes hypochlorhydria is the use of antacids or medicines that suppress stomach acid. Also autoimmune gastritis, where the immune system attacks the parietal cells leading to hypochlorhydria (low stomach acidity) is a possible cause. In this condition, hypochlorhydria results in an elevated gastrin level in an attempt to compensate for increased pH in the stomach. Eventually, all the parietal cells are lost and achlorhydria results to a loss of negative feedback on gastrin secretion. Other causes of hypergastrinemia are G-cell hyperplasia (overactivity of gastrin-producing cells in the stomach), Helicobacter pylori infection of the stomach, mucolipidosis type IV (5, 15) (**Table 4**).

HYPERGASTRINEMIA WITHOUT GASTRIC ACID HYPERSECRETION:
Atrophic gastritis (with or without pernicious anemia) Gastric cancer without involvement of the gastric antrum Therapy with H-2 blockers or proton pump inhibitors (PPIs)
HYPERGASTRINEMIA WITH GASTRIC ACID HYPERSECRETION:
Gastrinoma Antral G cell hyperplasia Duodenal ulcer Gastrojejunostomy or Billroth II Pyloric stenosis Hypercalcemia Massive bowel resection Chronic renal impairment

Table 4. Conditions associated with hypergastrinemia

3.4 VIP

Vasoactive intestinal peptide (VIP) is a peptide hormone containing 28-amino acid residues. It is produced in many areas of the human body including the gut, pancreas and suprachiasmatic nuclei of the hypothalamus in the brain.

In normal physiology VIP acts as a neuromodulator and not as an hormone, since it circulates in low quantity even an increase of about 20-50% of normal reference range is significant. VIP is released from neurons, peripheral ganglia, throughout the GI tract, in the urogenital system, respiratory tract and blood vessels. VIP has several effects on the digestive system:

it relaxes the lower esophageal sphincter ,the fundic smooth muscle and suppress gastric acid secretion. These effects work together to increase motility. Like secretin, it stimulates secretion of water and bicarbonate and stimulates secretion of chloride and water from large intestine; in small intestine inhibits absorption and the contractile effect of CCK. Moreover it enhances the release of insulin and glucagon. VIP has also significant effects on the cardiovascular system. It causes coronary vasodilation as well as it has a positive inotropic and chronotropic effect. VIP helps to regulate prolactin secretion (2).

VIPoma is much less common that insulinoma and gastrinoma with an incidence of approximately 0.02 per 100,000 per year. VIPoma is characterized by watery diarrhea, hypokalemia and achlorhydria (WDHA syndrome or pancreatic cholera syndrome, or also called Verner Morrison syndrome). Due to VIP effects as a potent stimulator of intestinal secretion and inhibitor of gastric acid secretion, the massive amounts of secreted VIP cause profound and chronic **w**atery **d**iarrhea (fasting stool volume > 750 to 1000 mL/day) and resultant dehydration, **h**ypokalemia, **a**chlorhydria (hence WDHA-syndrome), acidosis, vasodilation (flushing and hypotension), hypercalcemia and hyperglycemia. The watery diarrhea may be intermittent at the onset , but it may rapidly escalate and reach a volume of 15-20L per day, causing profound alteration in fluids and electrolytes control. Hypokalemic acidosis is due to bicarbonate and potassium loss across the bowel mucosa; it may provoke asthenia and tetanic contraction. Gastric achlorhydria occurs only in 50% of patients, while hypochlorhydria is usually present. Abdominal pain and weight loss are also common features. Other signs are hypercalcemia, related to VIP direct action on bone metabolism, and flushes that may cause some confusion with classical midgut carcinoid syndrome (5) (see above, **Table 3**).

The majority of VIPomas occurs in the pancreas, while about 10-15% arises in the ganglionic chain and most common in the adrenal medulla. In children, besides, VIP-producing tumors may occur in ganglioneuroma and neuroblastoma. About 50-60% of VIP-secreting tumors are malignant and present hepatic involvement. It may arise in contest of MEN1 syndrome (2).

3.5 Glucagon

Glucagon is a hormone secreted by alpha cells (α-cells) of the islets of Langerhans of the pancreas and from the L cells in the intestinal mucosa. Glucagon is a 29-amino acid polypeptide and its main action is to raise blood glucose levels. From these two sites, proglucagon is processed differently. In the pancreas, proglucagon is processed to produce glucagon, glycentin-related peptide, intervening peptide, and the major glucagon fragment. Intestinal proglucagon undergoes alternative posttranslational processing that generates glycentin, sometimes referred to as gut glucagon, glucagon-like peptide 1 (GLP1), and glucagon-like peptide 2 (GLP2) (2).

Plasma glucagon is a specific marker for Glucagonoma. Glucagonoma occurs at approximately the same frequency as VIPoma. Circulating glucagon concentrations are typically more than 5-fold higher than the reference range. Both pancreatic glucagon and glycentin are measured in high concentrations. Considering the importance of glucagon in the control of blood glucose, one would expect a glucagon-secreting tumor to produce a profound syndrome. However this is not the case and glucagonoma usually presents late

with extensive metastatic spread, mild diabetes, and a characteristic rash (necrolytic migratory erythema) (5).

Increased secretion of glucagon is also caused by other causes, like decreased plasma glucose (indirectly), increased catecholamines - norepinephrine and epinephrine, increased plasma amino acids (to protect from hypoglycemia if an all-protein meal is consumed), sympathetic nervous system, acetylcholine, cholecystokinin. Decreased secretion of glucagon is caused by somatostatin, insulin, increased free fatty acids and keto acids into the blood, increased urea production (16).

3.6 Somatostatin

Somatostatin is a peptide hormone that regulates the endocrine system and affects neurotransmission and cell proliferation. Somatostatin is classified as an inhibitory hormone, that exerts its effects mainly on anterior pituitary, by inhibiting the release of GH, opposing the effects of Growth Hormone-Releasing Hormone (GHRH), and inhibiting the release of thyroid-stimulating hormone (TSH) and on gastrointestinal system, by suppressing the release of other gastrointestinal and pancreatic hormones, decreasing gastric emptying, and reducing smooth muscle contractions and blood flow within the intestine (2).

Pancreatic somatostatinoma is a tumor of the delta cells of the endocrine pancreas that produces somatostatin. It is uncommon and symptoms are vague, thus diagnosis is frequently delayed. Clinical syndrome is characterized by a triad of : mild diabetes mellitus, steatorrhea and gallstones. Also hypochlorhydria can be associated. Patients may present with clinical endocrine syndrome due to elevated somatostatin that can cause diabetes mellitus, by inhibiting insulin secretion, steatorrhea by inhibiting cholecystokinin and secretin, gallstones by inhibiting cholecystokinin which normally induce gallbladder myocytes contraction, and hypochlorhydria caused by inhibiting gastrin, which normally stimulates acid secretion. However, more frequently patients present with symptoms related with tumor bulk. In presence of a somatostatinoma circulating somatostatin concentrations may be more than 100 times the reference range (2, 5).

3.7 Other circulating markers

3.7.1 Corticotropin-releasing hormone (CRH)

Corticotropin-releasing hormone, a 41-amino acid peptide derived from a 191-amino acid preprohormone, acts as hormone and neurotransmitter in the stimulation of pituitary synthesis of ACTH in stress response. In normal physiology CRH is produced by parvocellular neuroendocrine cells (contained within the paraventricular nucleus of hypothalamus).

Ectopic CRH production is rare, it may occur in patients with medullary thyroid carcinoma (about 33%) and pheochromocytoma (19%), carcinoid (5%) and small cell lung carcinoma (about 10 %) and prostate cancer (17). The main clinical feature is Cushing 's syndrome; levels of cortisol are elevated (>900 nmol/l) as ACTH, DHEA-S. Overnight administration of dexamethasone doesn't suppress cortisol secretion.

3.7.2 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, and is carried by the hypothalamic-hypophyseal portal system to anterior pituitary gland, where stimulates growth hormone (GH) secretion. Hypothalamic tumors, including hamartomas, choristomas, gliomas, and gangliocytomas, may produce excessive GHRH with subsequent GH hypersecretion and resultant acromegaly. Peripheral GHRH levels are not elevated in patients with hypothalamic GHRH-secreting tumors, supporting the notion that excess eutopic hypothalamic GHRH secretion into the hypophyseal portal system does not appreciably enter the systemic circulation. Excessive ectopic peripheral production of GHRH is present in several tumors, including carcinoid tumors, pancreatic cell tumors, small-cell lung cancers, adrenal adenomas, and pheochromocytomas, which have been reported to secrete GHRH. In these cases, peripheral GHRH levels are elevated even if acromegaly in these patients, is uncommon. For these reasons measuring GHRH plasma levels provides a precise and cost-effective test for the diagnosis of ectopic acromegaly. Elevated circulating GHRH levels, a normal or small-size pituitary gland, or clinical and biochemical features of other tumors known to be associated with extra-pituitary acromegaly, are all indications for extra-pituitary production of GHRH (18).

3.7.3 Calcitonin

Calcitonin is a 32-amino acid peptide released, in normal physiology, only in non-follicular C-cells of the thyroid . It is produced as a 136-amino acid precursor (pro-calcitonin) and processed in secretory granules to the active form. The synthesis and release of calcitonin are closely related to calcium serum levels. Calcitonin is raised in medullary thyroid cancer, where concentration may be thousands-fold the reference range. Medullary thyroid cancers frequently arise as part of multiple endocrine neoplasia type 2 (MEN2) syndrome. Calcitonin may also be raised in some pancreatic neuroendocrine tumor, especially those that are multi-hormone producing and bronchial carcinoid. Usually ectopic-produced calcitonin is a large molecule without biochemical activity (2).

4. Provocative tests

4.1 Insulinoma: 72-hour fast

NETs secreting insulin are termed insulinomas and are almost exclusively intrapancreatic in nature. Insulinomas secrete proinsulin, insulin and C-peptide intermittently, and, although insulin concentrations in the circulation may often be within reference range, insulin is at most times inappropriately high for the blood glucose concentration.

The diagnosis is suggested in the presence of the Whipple's triad: symptoms of hypoglycemia, glucose < 2.5 mmol/l (45 mg/dl) and relief of symptoms with administration of glucose. Hypoglycemia-induced clinical signs are classically present in the early morning pre-prandial phase or maybe exercise-induced. The typical signs are due to activation of adrenergic nervous system (palpations, sweating pallor, anxiety) and neuroglycopenia (personality changes, and loss of consciousness). The latter symptom reflects both the severity and duration of hypoglycemia. Although these symptoms are profound, they may

be intermittent and diagnosis is not always straightforward. The 72-hour fast is the gold standard for diagnosing insulinoma and it attests autonomous insulin secretion and the failure of appropriate insulin suppression in the presence of hypoglycemia. In fact, a carefully supervised 72-hour starvation usually precipitates hypoglycemia within the first 36 to 48 hours. A 72-hour period is universally recognized as the most appropriate duration although some groups have proposed a shorter fast of 48 h. Symptoms appear within 12 h for one third of patients, 80% within 24 h, 90% with 48 h and approaching 100% within 72 h. Absolute values of glucose and insulin are the most important variables and any measurable insulin is abnormal when blood glucose drops to 2.5 mol/l (45 mg/dl). The patient should be monitored in a supervised environment and fasting should be accompanied by an intravenous line. Absolute blood (venous) determinations should be performed at least 2–4 times per day and bedside measurements can be used in the presence of clinical symptoms to determine if more definitive measurements should be made. Blood should also be drawn for insulin measurement concurrently with glucose estimations, and assay for insulin and C-peptide when the hypoglycemia is confirmed. The differential diagnosis is insulin abuse (known insulin-requiring diabetes or factitious hypoglycemia): it can be detected when insulin is increased but not pro-insulin or C-peptide, so the measurement of increased pro-insulin or C-peptide secures the diagnosis of insulinoma. On rare occasions the abuse of sulphonylureas and related insulin secretagogues result in a clinical picture similar to patients with insulinoma and may be diagnosed only by a positive drug screen. Even patients on regular therapy with oral hypoglycemic medications in the setting of renal impairment may show symptoms that mimic insulinoma (5, 7).

4.2 Gastrinoma: Secretin test

Gastrinoma is the second most common secretory pancreatic NET with just more than half malignant at presentation. Gastrinoma is the most common GEP NET associated with MEN1, with approximately 25-35%of gastrinomas being associated with MEN1. Gastrinomas may be found in the duodenum (50-70%) and less commonly in the pancreas (20-40%) and secrete excess of gastrin, leading to ulceration in the stomach, duodenum and the small intestine. Hydrochloric acid (HCl) also causes hyperperistalsis and inhibits the activity of lipase causing severe diarrhea. The clinical syndrome is therefore characterized by the classical triad of gastric acid hypersecretion, severe peptic ulceration, and non-beta cell islet tumor of pancreas (gastrinoma) and this is called Zollinger Ellison syndrome (ZES). Despite a clear clinical syndrome, the primary gastrinoma may be often smaller than can be visualized by any current radiological method. Therefore, circulating gastrin remains a useful tool for diagnosis. The diagnosis of ZES can be established by the demonstration of elevated fasting serum gastrin (FSG) in the presence of low gastric pH (<5.0). In fact, in the presence of gastric acid (pH<5.0), a serum gastrin value greater than 1000 pg/mL is virtually diagnostic of the disorder. However, about two-thirds of patients with the Zollinger-Ellison syndrome have serum gastrin concentrations less than 10 times the upper limit of normal (generally between 150 and 1000 pg/mL), so FSG alone is not adequate for a conclusive diagnosis of ZES. Moreover hypergastrinemia can be seen in patients with achlorhydria associated with chronic atrophic fundus gastritis (e.g., pernicious anemia), proton pump inhibitor drugs and in other conditions with

hyperchlorhydria (e.g., Helicobacter pylori infection, gastric outlet obstruction, renal failure, antral G cell syndromes, short bowel syndrome, retained antrum) (see above **Table 4**) (2).

The secretin stimulation test can differentiate patients with gastrinomas from those with hypergastrinemia of different etiologies and identify those patients with gastrinoma and only mild hypergastrinemia (**Figure 2**). Secretin is normally secreted by duodenal S-cells in response to a low luminal pH following food-stimulated acid secretion. Its primary action is to cause the release of bicarbonate rich pancreatic juice from pancreatic acinar cells, thus neutralizing the acidic juice delivered from the stomach. Under physiological conditions, secretin decreases antral gastrin secretion. However in presence of a gastrinoma, secretin stimulates the release of stored gastrin by gastrinoma cells, and therefore most patients with these tumors have a dramatic rise in serum gastrin in response to a secretin infusion. In contrast, normal gastric G cells are inhibited by secretin, and therefore serum gastrin concentrations do not rise in patients with other causes of hypergastrinemia. The secretin stimulation test is performed by administering 2 U of secretin/kg body weight intravenously over one minute; a baseline serum gastrin is measured twice before the secretin is administered and 2, 5, 10, 15, and 20 minutes later. Several criteria have been proposed to define a positive test; the most commonly accepted one is a rise in serum gastrin by 200 pg/mL (95 pmol/L) or more, which is diagnostic for the presence of gastrinoma in more than 90% of cases. The use of this test has recently been re-evaluated, suggesting a lower cut-off (a rise >120 pg/mL in serum gastrin concentration) to define a positive test, thus obtaining an increase in the sensitivity (94%) without loss of specificity (100%) (7, 15).

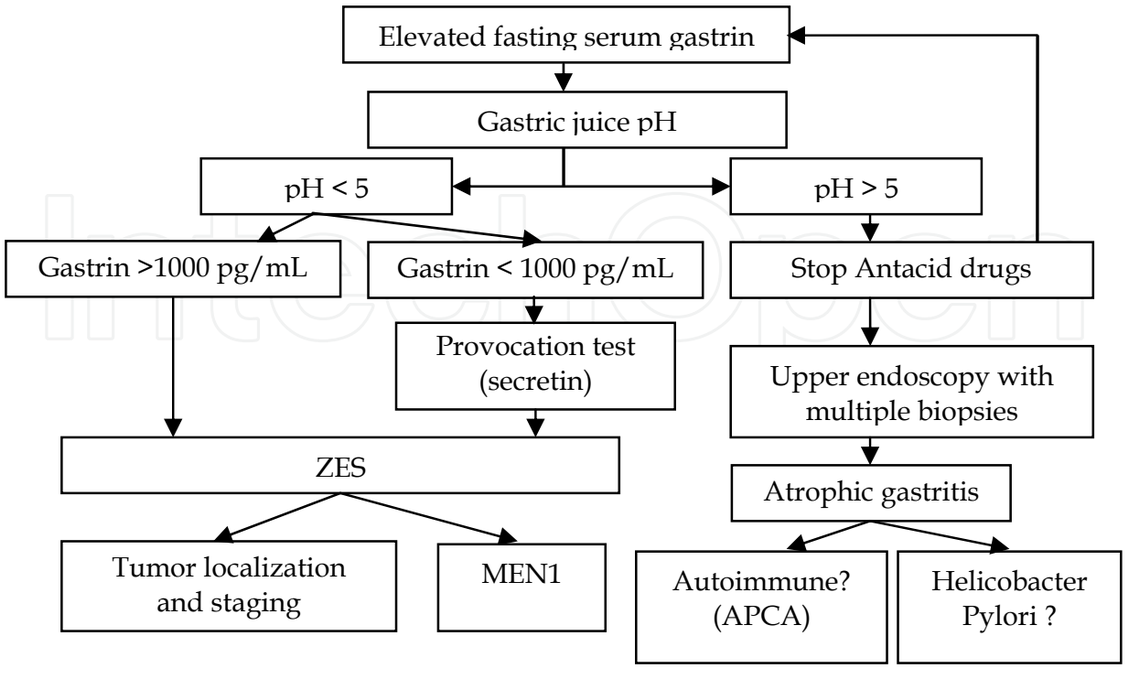

Fig. 2. Suggested algorithm for the investigation of hypergastrinemia.

Another critical point regarding secretin test is to establish whether the the same diagnostic operative characteristics of the test remains unchanged even during treatment with inhibitors of acid secretion. In fact the standard secretin test was performed with patients off antacids and anticholinergics for at least 12 hours and H2 antagonists and proton pump inhibitors were not available at the time the test was described. However, patients with severe ZES can develop complications (such as acute bleeding and perforation) if acid suppression is discontinued. Thus, discontinuation of acid suppression should be performed cautiously. While the accuracy of the test in patients taking proton pump inhibitors (PPIs) has not been well established, some authors suggest that PPIs do not interfere with the interpretation of the secretin stimulation test and that PPIs do not need to be discontinued. However, the leading experts suggest PPIs should be discontinued for one-two weeks (1, 7, 10).

The secretin stimulation test has also been shown to be a valuable predictor of recurrence of gastrinoma following surgery (15).

4.3 Other provocative tests for gastrinoma

In addition to the secretin test, several other stimulation tests have been developed to attempt to differentiate between neoplastic and non-neoplastic causes of hypergastrinemia. These include calcium, meal and glucagon stimulation tests.

4.3.1 Calcium stimulation test

The calcium provocation test is based on the principle that calcium administration stimulates the release of stored gastrin from gastrinoma cells, as well as for secretin test. Serum gastrin concentrations are measured at 0, 30, 60, 90, 120, 150, 180 minutes intervals during an intravenous infusion of 10% calcium gluconate (5 mg/kg body weight over 3 h). More than 80% of gastrinoma patients show an increase in serum gastrin of >200 pg/mL within the third hour of calcium infusion, usually with a positive response at 120 to 180 min. The sensitivity of this test is low (about 43%), thus it can't replace the secretin test, but it could be used in patients with an high clinical suspicion of ZES and a negative secretin test (15).

4.3.2 Meal stimulation test

The physiological response to a standard test meal (of two eggs and toast) is an increase in plasma gastrin of up to two to threefold. Patients with ZES demonstrate no or only a very minimal increase in serum gastrin concentration after ingestion of such a protein rich test meal, whereas patients with other causes of hypergastrinemia show a more pronounced increase in serum gastrin following the same protein test meal. However, data on these results seems to be conflicting and a significant overlap in patients with ZES and with other antral syndromes is observed, thus the meal stimulation test for investigation of the cause of hypergastrinemia is not currently recommended (15).

4.3.3 Glucagon stimulation test

Glucagon stimulation test consists in a rise in serum gastrin concentration following the administration of glucagon to patients with ZES, but a paradoxical fall in serum gastrin

concentrations in patients with pernicious anemia. This test has not been widely evaluated; however, further studies are required to determine the overall accuracy of the glucagon stimulation test (15).

5. Conclusions

Numerous biochemical markers have been identified in association with GEP-NETs, but few have the specificity or predictive value of CgA or urinary 5-HIAA, and their measurement is complex. Recent studies have proposed that alkaline phosphatase and neurokinin A are better predictors of survival in metastatic NETs than CgA but additional rigorous data to support this assertion are required.

Circulating biomarkers offer a useful diagnostic tool in conjunction with radiology and tissue pathology for NETs. However, these biomarkers are more reliable when used to monitor disease progression, response to treatment, and for early detection of recurrence after treatment.

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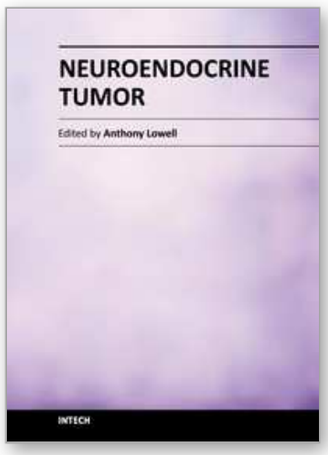

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