REVIEW

Chromogranin A as circulating marker for diagnosis and management of neuroendocrine neoplasms: more flaws than fame

Vincenzo Marotta[1],*, Maria Chiara Zatelli[2],*, Concetta Sciammarella[3], Maria Rosaria Ambrosio[2], Marta Bondanelli[2], Annamaria Colao[1] and Antongiulio Faggiano[4]

[1]Department of Clinical Medicine and Surgery, Federico II University, Naples, Italy
[2]Section of Endocrinology and Internal Medicine, Department of Medical Sciences, University of Ferrara, Ferrara, Italy
[3]IOS & COLEMAN Srl, Naples, Italy
[4]Thyroid and Parathyroid Surgery Unit, Istituto Nazionale per lo Studio e la Cura dei Tumori 'Fondazione G. Pascale' – IRCCS, Naples, Italy

Correspondence should be addressed to V Marotta: vinc.endo@libero.it

*(V Marotta and M C Zatelli contributed equally to this work)

Abstract

Owing to the heterogeneity of neuroendocrine neoplasms (NENs), the availability of reliable circulating markers is critical for improving diagnostics, prognostic stratification, follow-up and definition of treatment strategy. This review is focused on chromogranin A (CgA), a hydrophilic glycoprotein present in large dense core vesicles of neuroendocrine cells. Despite being long identified as the most useful NEN-related circulating marker, clinical application of CgA is controversial. CgA assays still lack standardization, thus hampering not only clinical management but also the comparison between different analyses. In the diagnostic setting, clinical utility of CgA is limited as hampered by (a) the variety of oncological and non-oncological conditions affecting marker levels, which impairs specificity; (b) the fact that 30–50% of NENs show normal CgA, which impairs sensitivity. Regarding the prognostic phase, there is prospective evidence which demonstrates that advanced NENs secreting CgA have poorer outcome, as compared with those showing non-elevated marker levels. Although the identification of cut-offs allowing a proper risk stratification of CgA-secreting patients has not been performed, this represents the most important clinical application of the marker. By contrast, based on prospective studies, the trend of elevated circulating CgA does not represent a valid indicator of morphological evolution and has therefore no utility for the follow-up phase. Ultimately, current knowledge about the role of the marker for the definition of treatment strategy is poor and is limited by the small number of available studies, their prevalent retrospective nature and the absence of control groups of untreated subjects.

Key Words
- ► chromogranin A
- ► neuroendocrine neoplasm
- ► biomarker
- ► diagnosis
- ► prognosis
- ► response to treatment

Introduction

Despite being rare diseases, neuroendocrine neoplasms (NENs) have shown a worldwide increase in the past several decades, with incidence rates rising from 1.52 to 7.41 cases per 100,000 from 1973 to 2012 (Leoncini *et al.* 2017). Therefore, physicians dealing with NENs urgently need better guidance as to clinical management, which is

still empiric (Faggiano *et al.* 2012, Oberg 2012). Actually, the definition of NENs gathers a heterogeneous group of diseases, including malignancies from several anatomic areas, such as stomach, intestine, rectum, pancreas, lung, adrenals and thyroid, and with variable evolution, from indolent to rapidly progressive (Baudin 2007, Yao *et al.* 2008a). The feature joining these tumors is that they arise from specialized cells spread throughout the body, belonging to the so-called diffuse neuroendocrine system, whose main ability is to produce, store and release the bloodstream bioactive molecules (Langley 1994, Kaltsas *et al.* 2004, Ferolla *et al.* 2008). Whether this biological activity produces characteristic syndromes represents the major factor affecting clinical scenario of NENs, which are accordingly classified into functional and non-functional (Kulke *et al.* 2012). Indeed, the former ones are usually diagnosed at an earlier stage because of endocrine symptoms related to the hormonal production, whereas the non-functional ones remain silent for large part of their natural history and are frequently diagnosed when metastases have already occurred (Modlin *et al.* 2008). Owing to these observations, a possible clinical application of tumor-related bioactive products, as detected in the serum or plasma, has represented the objective of a wide body of research. Particularly, researchers aimed to identify markers useful for (a) diagnosis anticipation and refining, (b) prognostic stratification and (c) disease evolution monitoring and response to treatment. Based on the relationship with codified hormone-related syndromes, circulating markers of NEN are differentiated in common or broad spectrum, including chromogranin A (CgA), pancreatic polypeptide and neuron-specific enolase, and specific or individual, including serotonin and its metabolite 5-hydroxyindolylacetic acid, gastrin, glucagon, insulin, C-peptide, vasoactive intestinal peptide, somatostatin, histamine, calcitonin, parathyroid, somatotropic, adrenocorticotropic hormones, catecholamines and their metabolites and neuropeptides (Ferolla *et al.* 2008). The present review is focused on CgA, a hydrophilic glycoprotein abundantly expressed in large dense core vesicles of neuroendocrine cells, whose main biological role is to regulate calcium-mediated exocytosis (Borges *et al.* 2010). Consistent with the definition of a common marker, elevated levels of circulating CgA have been associated with almost all types of NEN, including those arising from the gastroenteropancreatic tract and the bronchopulmonary area, which represent the majority, but also pheocromocytomas/paragangliomas, medullary thyroid carcinoma, Merkel cell carcinoma of the skin and (even if data are controversial) pituitary

and parathyroid adenomas (Sobol *et al.* 1986, Blind *et al.* 1992, Kimura *et al.* 1997, Nobels *et al.* 1997, Guignat *et al.* 2001, Tomassetti *et al.* 2001b, Campana *et al.* 2007, Zatelli *et al.* 2007). Despite having a long recognized role for the histological definition of NEN (Solcia *et al.* 2000), actual use of CgA as a circulating marker is far more tricky than expected (Modlin *et al.* 2014c). Indeed, the clinical utility of the test is affected by a variety of issues, which will be strictly analyzed in our review.

CgA physiology: production and biological functions

CgA belongs to the granin family, which also includes chromogranin B, secretogranins II and III and other proteins (7B2, NESP55, proSAAS and VGF). All of them are involved in a series of biological pathways controlling protein (peptides, hormones, neurotransmitters and growth factors) secretion upon secretagogue stimulation (Arvan *et al.* 1991). Besides being stored into secretory vesicles, the members of the granin family have many common properties, such as a similar acidic isoelectric point, the capacity to bind calcium ions and the ability to form aggregates. Furthermore, their structure typically includes multiple dibasic cleavage sites, which allow the processing into smaller peptides, each displaying a differential function (O'Connor & Frigon 1984, Gerdes *et al.* 1988, Borges *et al.* 2010, Mahata *et al.* 2010, Sanchez-Margalet *et al.* 2010, Helle & Corti 2015). Human CgA is encoded by the *CHGA* gene, located on chromosome 14q32.12. This 12.192 base-pair-long gene, encompassing 8 exons and 7 introns, is transcribed into a 2.041 base-pair-long mRNA, which is in turn translated into a 439-amino-acid mature protein (Winkler & Fischer-Colbrie 1992) showing 10 dibasic sites for proteolytic cleavage (Konecki *et al.* 1987). CgA-derived peptides include vasostatins (VST I: hCgA1–76 and VST II: hCgA1–115) (Aardal *et al.* 1993), pancreastatin (PST: hCgA357–428) (Tatemoto *et al.* 1986), catestatin (CST: hCgA352–372) (Mahata *et al.* 1997), a 14-amino-acid peptide with N-terminal tryptophan and C-terminal glutamic acid (hCgA324–337) and serpinin (bCgA 403–428) (Koshimizu *et al.* 2010). As shown in Fig. 1, CgA is synthesized at the rough endoplasmic reticulum, where it is inserted via the N-terminal signal peptide, and then transported to the Golgi complex (Kuehn *et al.* 1998). CgA is then packaged together with other secretory proteins (i.e. hormones and peptides) into immature granules, where it may be cleaved into the various derived peptides by specific processing enzymes. Upon acidification, secretory granules mature, thus
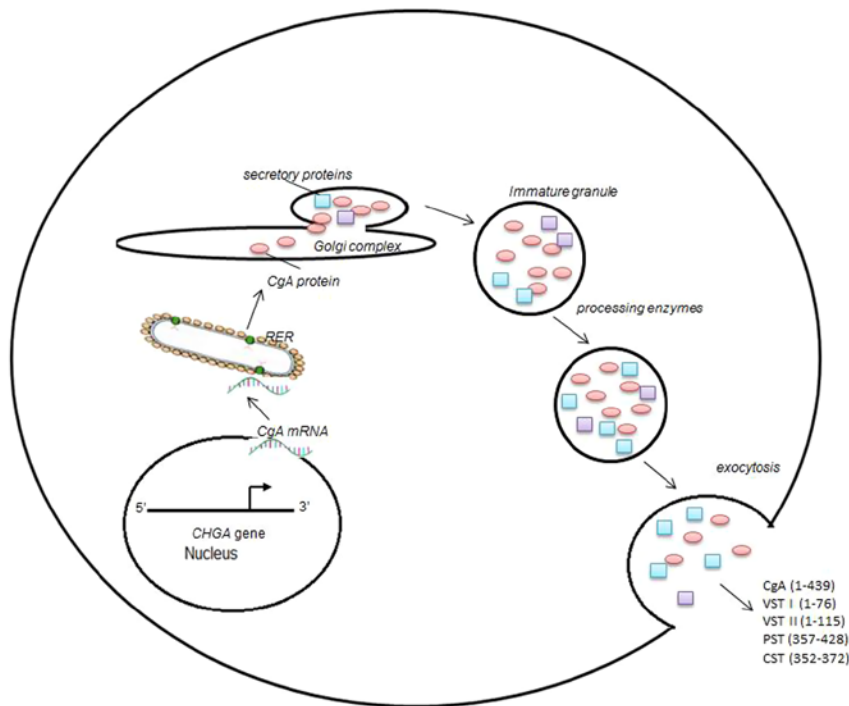
Figure 1
Physiology of production and secretion of CgA and related products.

becoming ready for stimulation-induced release (Kim *et al.* 2006). CgA and the derived peptides display several biological functions. VST I (1–76) and VST II (1–115) have vasodilator and antimicrobial properties. VST I has also been demonstrated to inhibit PTH secretion, promote cell adhesion and inhibit VEGF-induced endothelial cell proliferation/migration (Ferrero *et al.* 2004, Blois *et al.* 2006, Belloni *et al.* 2007). Furthermore, it promotes calcium entry into neutrophils (Zhang *et al.* 2009), indicating an immune-endocrine crosstalk. PST (357–428) induces hyperglycemia by inhibiting glucose-stimulated insulin release from β-cells (Tatemoto *et al.* 1986) and glucose uptake in adipocytes and hepatocytes (Gonzalez-Yanes & Sanchez-Margalet 2000) and by stimulating glucagon secretion and glycogenolysis (Sanchez-Margalet *et al.* 1992*a*,*b*). In addition, it inhibits PTH release and stimulates histamine release. CST (352–372) is a potent endogenous antagonist of the nicotinic cholinergic receptor, being able to inhibit nicotine-induced catecholamine secretion (Mahata *et al.* 1997, Mahata *et al.* 2004). CST has also been demonstrated to inhibit lypolysis and fatty acid oxidation by regulating adrenergic and leptin signaling (Borges *et al.* 2013). Due to its capacity to stimulate histamine release, CST acts as a potent vasodilator (Kruger *et al.* 2003). Furthermore, it was found to induce endothelial cell proliferation/migration and to reduce cardiac contractility. Intact CgA (1–439) controls dense core granule biogenesis as well as sorting and secretion of other proteins. Specifically, it prevents uncontrolled osmotic swelling of secretory vesicles, functioning as a matrix condenser for soluble intra-vesicular component (Borges *et al.* 2013). CgA has been demonstrated to participate in the regulation of cytosolic calcium stores, granule exocytosis in secretory cells (Yoo 2010, Yoo *et al.* 2010) and prohormone convertase activity. In addition, it is involved in blood pressure regulation through the stimulation of the sympathetic tone (Takiyyuddin *et al.* 1991, Dimsdale *et al.* 1992). Notably, CgA processing into CST induces opposite effects on blood pressure, since CST inhibits catecholamine secretion (as already discussed). This is a paradigm of the complexity of biological effects related to CgA, which depends on the balance between the intact and the cleaved protein.

Methods for circulating CgA determination

CgA detection is based on different assays, which are non-standardized and provide different information. This hampers both clinical management, where the same assay should be used from diagnosis to follow-up of each patients, and also comparison between different studies.

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: Minamiki *et al.* (2016) demonstrated highly sensitive CgA detection by means of an extended-gated organic-field-effect-transistor-based immunosensor, which employed a non-labeled monoclonal anti-CgA antibody. CgA may be assessed in plasma or serum, depending on the assay. Actually, a study by Woltering *et al.* (2006) showed that plasma and serum CgA levels, both measured by a two-site chemiluminescence immunometric assay using a biotinylated monoclonal antibody, displayed strong positive linear correlation ($r=0.9858$, $P<0.0001$), thus suggesting that the measurement could be performed in both sample types with consistent results. Although plasma CgA levels were significantly higher as compared with serum determination due to the matrix effect of each biological material, Glinicki *et al.* (2015), who used the CisBio IRMA, also found a good correlation between the 2 sample types ($r=0.8493$; $P<0.01$). More importantly, each assay is performed by using different antibodies, with varying sensitivity and specificity. As a consequence, different kits may lead to significantly different results, hampering the possibility of pooling and/or comparing data obtained by different research centers with different assays (Gut *et al.* 2016). Indeed, results from antibody-dependent assays are strikingly influenced by the employed antibodies. It has been reported that three different ELISA assays display different specificities for full-length CgA and its fragments, due to the use of the same capture antibody against CgA or VST I N-terminal regions coupled with three different detection antibodies against epitopes located in the central region of CgA, or against the six C-terminal residues of full-length CgA, or the six C-terminal residues of VST I. Indeed, these assays could detect intact and processed CgA, only intact CgA or only VST I, respectively (Helle & Corti 2015). Therefore, the detection of intact/cleaved CgA depends on the employed antibody. Sensitivity and specificity of available methods have been compared by a number of studies. Stridsberg *et al.* (2003) came to the conclusion that the best compromise between sensitivity and specificity is the use of RIA. On the other hand, a prospective multicenter study demonstrated that ELISA and IRMA methods display a good diagnostic performance, providing results that are comparable and showing a satisfactory correlation ($r=0.843$, $P<0.0001$) (Leon *et al.* 2005). Nevertheless, authors also reported a 36% discordance rate between the two methods, confirming previous findings (Ferrari *et al.* 2004) and suggesting that they might provide

partially different information. These results were further strengthened by a multicenter study comparing a two-step IRMA (IRMA; CGA-RIA CT, CisBio international-Shering, Gif-sur-Yvette, France) and an ELISA assay (DAKO Cytomation, Glostrup, Denmark). The employed IRMA assay was based on two monoclonal antibodies raised against CgA-unprocessed central domain (CgA 145–245), thus detecting total human CgA. On the other hand, the employed ELISA assay measured more CgA fragments, since it was based on two polyclonal rabbit antibodies directed toward a 23-kDa C-terminal CgA fragment. Results from the two assays were found to correlate and a receiving-operator characteristic (ROC) analysis found a cut-off of 53 ng/mL for IRMA and 16 U/L for ELISA as discriminating between controls and patients with active gastroenteropancreatic NENs (sensitivity 71.3 and 84%; specificity 71 and 85%, respectively) (Zatelli *et al.* 2007). An emerging CgA assay is the TRACE (KRYPTOR), a sandwich immunofluorescent method using two mouse monoclonal antibodies, based on a non-radioactive energy transfer between a donor (europium cryptate) and an acceptor (XL665). This assay has been recently compared with a widely used ELISA kit, the DAKO (Wolf *et al.* 2014). Authors showed an excellent correlation between serum samples analyzed with the first-generation KRYPTOR and those measured with the ELISA in EDTA plasma ($r=0.99$). The workflow of the KRYPTOR was reported to be much faster than the ELISA, but it seems to be more sensitive to the storage temperature of the samples. Furthermore, the KRYPTOR assay issues different CgA levels depending on the sample origin (higher when starting from serum as compared to plasma), suggesting a possible interference by other analytes. Despite these limitations, the sensitivity of the method is promising since Popovici *et al.* (2014) showed values of 100 and 94% in pheochromocytoma/paraganglioma and GEP NENs, respectively. A more recent study compared the results of the KRYPTOR assay with those of a solid-phase ELISA assay (CisBio) in serum samples (Van der Knaap *et al.* 2015). Authors found that CgA levels measured with the KRYPTOR method were significantly higher as compared to those measured by the ELISA CisBio, independently of gender, use of proton pump inhibitors (PPIs), renal function, referral department and tumor location. However, storage at low temperature ($-20°C$) confirmed to be crucial for the analyte recovery, also indicating a low stability of the assay with time, which leads to progressive decay in CGA concentrations. This issue has been addressed by developing a second-generation assay, which uses two monoclonal antibodies recognizing different CgA epitopes, with a reduced

Table 1 Patients features, methodological approach and results from studies assessing circulating CgA as a diagnostic marker of NEN.

Study (year)	NEN cohort			Comparison group	Interfering diseases	PPIs assumption	Assay	Cut off values	Sensitivity (%)	Specificity (%)
	N	Primary location	Stage IV (N)							
Tomassetti et al. (2001a,b)	80	GEP	NA	28 HS	Excluded	Not considered as exclusion criterium	ELISA	17 U/L	56	100
Bernini et al. (2001)	48	All sites	NA	130 HS 48 HP	Excluded	Not considered as exclusion criterium	RIA	100 ng/mL	75	100 97.9
Peracchi et al. (2003)	61	GEP	28	50 HS	Not considered as exclusion criteria	Not considered as exclusion criterium	ELISA	20 U/L	92	83
Nehar et al. (2004)	124	GEP	97	50 HS	Excluded	Not considered as exclusion criterium	IRMA	100 µg/L	66	100
Zatelli et al. (2007)	81	GEP	NA	129 HS	Excluded (with the exception of CAG)	Excluded	IRMA ELISA	53 ng/mL 16 U/L	71.3 71	84 85
Campana et al. (2007)	238	GEP, lung	95	48 HS 42 CAG	Excluded	Excluded –	ELISA	18 U/L 53 U/L	85.3 66.5	95.8 71.4
Belli et al. (2009)	119	GEP	84	39 HS	Excluded	Excluded	RIA	2.8 nmol/L	92.3	100
Donica et al. (2010)	41	All sites	30	15 HS	Not considered as exclusion criteria	Not considered as exclusion criterium	ELISA	18 U/L	71	87
Molina et al. (2011)	66	All sites	45	52 HS 98 IDs	Excluded –	Not considered as exclusion criterium –	IRMA ELISA RIA IRMA ELISA RIA	90 ng/mL* 60 ng/mL* 6 nmol/L* 90 ng/mL* 60 ng/mL* 6 nmol/L*	80.3 83.3 65.2 80.3 83.3 65.2	100 100 100 35.7 46.9 31.6
Vezzosi et al. (2011)	184	GEP	44	No comparison group	Not considered as exclusion criteria	Not considered as exclusion criterium	IRMA	98.1 ng/mL	71	Not available
Marotta et al. (2012)	42	All sites	8	100 HS (benign nodular goiter)	Not considered as exclusion criteria	Not considered as exclusion criterium	IRMA	Not identified	Not defined (failed ROC analysis)	Not defined (failed ROC analysis)
Korse et al. (2012)	573**	All sites	388	282 HS	Not considered as exclusion criteria	Not considered as exclusion criterium	RIA	87 µg/L	69	95
Modlin et al. (2013)	81	GEP	NA	94 HS	Not considered as exclusion criteria	Not considered as exclusion criterium	ELISA	19 U/L	32	99
Tohmola et al. (2014)	41	All sites	NA	26 HS	Not considered as exclusion criteria	Not considered as exclusion criterium	RIA	6 nmol/L	51	86

CAG, chronic atrophic gastritis; GEP, gastroenteropancreatic; HP, hypertension; HS, healthy subjects; IDs, interfering diseases; N, number; NEN, neuroendocrine neoplasm; PPIs, proton-pump inhibitors; ROC, receiving-operator characteristics.
*Cut-off values extrapolated from HS to obtain 100% specificity; **the cohort included a majority (293 cases) of grade 3 patients.

impact of protein folding on CgA measurement (Ferraro *et al.* 2016). Utility of this improved method has also been demonstrated in studies exploring the value of CgA as a predictive marker in tumors different from NENs (Niedworok *et al.* 2017). However, the application of this method in real-life clinical practice is still poor, so it does not impact in management of NENs at present.

Circulating CgA in the diagnostic phase of NEN

The definition of CgA metrics in NEN diagnostics is not univocal, as hampered by the wide variability of analytical approaches applied by the various studies.

The most critical discrepancies were (a) the composition of the case groups, with the strongest difference being represented by the fact that some studies included NEN from all sites (Bernini *et al.* 2001, Donica *et al.* 2010, Molina *et al.* 2011, Korse *et al.* 2012, Marotta *et al.* 2012, Tohmola *et al.* 2014), whereas some others tried to analyze more homogeneous set of patients, mainly selecting gastroenteropancreatic tumors (Tomassetti *et al.* 2001*a*, Peracchi *et al.* 2003, Nehar *et al.* 2004, Zatelli *et al.* 2007, Belli *et al.* 2009, Modlin *et al.* 2013) (Table 1); (b) the composition of the control groups: although the majority of studies used healthy subjects as controls (Bernini *et al.* 2001, Tomassetti *et al.* 2001*b*, Peracchi *et al.* 2003, Nehar *et al.* 2004, Campana *et al.* 2007, Zatelli *et al.* 2007, Belli *et al.* 2009, Donica *et al.* 2010, Molina *et al.* 2011, Korse *et al.* 2012), which represents the best approach to assess the diagnostic performance of a marker (Shapiro 1999), some researchers determined the metrics of circulating CgA by comparing NEN with non-NEN tumors (Nobels *et al.* 1998, Panzuto *et al.* 2004) or active versus disease-free NEN (Bajetta *et al.* 1999, Panzuto *et al.* 2004); (c) the consideration of interfering factors: some authors tried

to clean up the control group from those conditions with known effect on CgA levels, thus obtaining a more pristine evaluation of marker specificity (Bernini *et al.* 2001, Tomassetti *et al.* 2001*b*, Nehar *et al.* 2004, Campana *et al.* 2007, Zatelli *et al.* 2007, Belli *et al.* 2009, Molina *et al.* 2011), whereas some others did not, thus providing data actually applicable into real-life practice (Peracchi *et al.* 2003, Donica *et al.* 2010, Korse *et al.* 2012, Marotta *et al.* 2012, Modlin *et al.* 2013, Tohmola *et al.* 2014) (Table 1).

Specificity

A wide range of conditions, both benign and malignant (Table 2), can induce NEN-unrelated CgA elevations, thus generating false-positive results (Ardill & O'Dorisio 2010). This strikingly hampers test specificity, which is considered as the major weakness of circulating CgA in the diagnostic setting of NEN (Kidd *et al.* 2016, Modlin *et al.* 2010*a*).

Non-oncological causes of CgA elevation

Real-life application of circulating CgA for NEN diagnostics is hampered by a variety of interfering non-oncological conditions, including benign diseases and iatrogenic causes, which are extremely common. These conditions strikingly affect test specificity and should mandatorily be considered by clinicians when interpreting CgA values.

Since secretion of CgA is ubiquitary (Lamberts *et al.* 2001), a variety of non-neoplastic processes inducing tissue damage and remodeling may produce elevations of the marker. These include a variety of gastrointestinal disorders, such as chronic atrophic gastritis (CAG) (Peracchi *et al.* 2005), Helicobacter pylori infection (Waldum *et al.* 1996), liver cirrhosis and chronic hepatitis (Spadaro *et al.* 2005), pancreatitis (Malaguarnera *et al.* 2009), inflammatory bowel diseases (Sciola *et al.* 2009)

Table 2 Conditions affecting CgA-circulating levels.

Non-oncological		Oncological
Benign diseases	Iatrogenic causes	
Gastrointestinal: chronic atrophic gastritis, Helicobacter pylori infection, liver cirrosi, chronic hepatitis, pancreatitis, inflammatory bowel diseases, irritable bowel	Proton pump inhibitors	Colorectal carcinoma
Cardiovascular: hypertension, heart failure, acute coronary syndromes	Histamine 2 receptor antagonists	Gastric carcinoma
Renal and hepatic dysfunctions	Serotonin reuptake inhibitors	Pancreatic carcinoma
Others: giant cell arteritis, rheumatoid arthritis, systemic lupus erythematosus, pulmonary obstructive disease, hyperthyroidism		Prostate carcinoma
		Breast carcinoma Hepatocellular carcinoma Ovarian carcinoma

and even irritable bowel syndrome (Sidhu *et al.* 2009). Among cardiovascular diseases, elevated CgA levels have been reported in hypertension, with higher levels being demonstrated in untreated patients (Takiyyuddin *et al.* 1995), chronic heart failure, where more accentuated elevations were detected in the fourth grade of the NHYA scale (Ceconi *et al.* 2002) and acute coronary syndromes, where higher concentrations predicted worsened outcome (Jansson *et al.* 2009). Other benign conditions increasing CgA levels include some rheumatoid diseases such as giant cell arteritis, rheumatoid arthritis and systemic lupus erythematosus (Di Comite *et al.* 2009*a*,*b*) and pulmonary obstructive disease (Hoshino *et al.* 2008). Due to reduced clearance, elevation of circulating CgA also occurs in the case of kidney and liver functional impairment (O'Connor *et al.* 1989). Particularly, the grade of renal dysfunction is directly related to CgA levels and may lead to concentrations as high as those detected in NEN patients (Hsiao *et al.* 1990). Ultimately, increased CgA levels have also been reported in endocrine disorders of non-neuroendocrine nature, such as hyperthyroidism, likely due to enhanced sympathetic activity which pairs with attenuation of the vagal tone (Al-Shoumer & Vasanthy 2009). The main iatrogenic cause of CgA elevation is the use of PPIs and other acid-blocking drugs, which are largely administered by physicians (Fossmark *et al.* 2008). Indeed, inhibition of gastric acid production leads to compensatory hypergastrinemia and G-cell hyperplasia, which in turn induce ECL–cell hyperplasia. Both G- and ECL-hyperplasia are responsible for CgA overproduction (Kuipers 2006). The role of the reported non-oncological conditions in affecting specificity of circulating CgA as a diagnostic marker of NEN emerges when comparing ROC analyses of studies trying to skim non-neoplastic controls for the presence of possible false-positive inductors with those not performing any selection (Table 1). Indeed, authors applying the former approach reported values ranging from 95 to 100% (Bernini *et al.* 2001, Tomassetti *et al.* 2001*b*, Nehar *et al.* 2004, Campana *et al.* 2007, Belli *et al.* 2009, Molina *et al.* 2011), with the only exception of Zatelli *et al.* (2007), who found 84/85% specificity (based on the detection method) likely due to the fact that CAG was not ruled out. By contrast, specificity was less than 90% in the majority of studies where exclusion of interfering conditions was not performed (Peracchi *et al.* 2003, Donica *et al.* 2010, Vezzosi *et al.* 2011, Marotta *et al.* 2012, Tohmola *et al.* 2014). Furthermore, some authors specifically assessed the effect of benign conditions on test specificity by comparing the same cohort of NENs with separate groups of healthy subjects and patients carrying

one or more interfering diseases (Table 1). Campana *et al.* (2007) selected a separate cohort of CAG patients reporting a drop, from 95.8 to 61.4%, of CgA specificity. More recently, Molina *et al.* (2011) analyzed a separate group of patients with renal failure, gastric diseases, heart failure, liver cirrhosis, hypertension and inflammatory bowel diseases, showing a reduction in test specificity, which dropped from 100% to less than 50%.

Oncological causes of CgA elevation

The actual impact of CgA elevation related to tumors other than NEN on test specificity is not univocal, due to the heterogeneity of available studies. However, the aim of future research should be to define performance of circulating CgA in differentiating NENs from those non-neuroendocrine malignancies posing an issue of differential diagnosis.

A variety of non-NEN malignancies are characterized by increased CgA levels (Glinicki & Jeske 2011). The majority of them present a histological pattern of neuroendocrine differentiation, including several digestive tumors, such as colorectal adenocarcinoma (Syversen *et al.* 1995), gastric and pancreatic cancer (Malaguarnera *et al.* 2009) and prostate adenocarcinoma (Angelsen *et al.* 1997). By contrast, there are some tumors showing CgA elevation where the presence of histological neuroendocrine differentiation has not been reported, such as primary hepatocellular cancer (Spadaro *et al.* 2005) and breast cancer (Giovanella *et al.* 2001). To date, the capability of circulating CgA in discriminating NEN from other malignancies has been evaluated by many studies, which reported controversial results characterized by wide variation of specificity values (Nobels *et al.* 1998, Nehar *et al.* 2004, Panzuto *et al.* 2004, Molina *et al.* 2011, Marotta *et al.* 2012) (Table 3). This was likely due to the heterogeneity of both NEN groups and neoplastic controls, with the latter including different tumor types. However, the mentioned papers do not provide a real picture of clinical practice, where what is actually required is to distinguish NENs from non-neuroendocrine malignancies posing to clinicians issues of differential diagnosis. This is particularly crucial for non-functional NENs, given the absence of the distinctive clinical and biochemical features related to hormone overproduction (Kulke *et al.* 2015). To date, poor data are available about this issue. Some authors focused on the possible role of circulating CgA in determining differential diagnosis between the pancreatic NEN and the various pancreatic malignancies, such as ductal adenocarcinoma, cystic tumors, solid pseudopapillary tumors, acinar cell carcinoma, squamous

Table 3 Composition of the study groups and results from studies assessing circulating CgA as a diagnostic marker between NEN and non-NEN tumors.

Study (year)	NEN group	Non-NEN group	Sensitivity (%)	Specificity (%)
Nobels *et al.* (1997)	211 from all sites	180: breast carcinoma, non-small cell lung cancer, pancreatic carcinoma, adenocarcinoma of unknown origin, non-Hodgkin lymphoma, Hodgkin lymphoma, multiple myeloma, meningioma, and astrocytoma	53	93
Panzuto *et al.* (2004)	68 GEP	24: gastric, colorectal, and pancreatic carcinoma	84	63
Nehar *et al.* (2004)	124 GEP	77: thyroid carcinoma, non-endocrine pancreatic tumors, others unspecified	62.9	97.4
Molina *et al.* (2011)	66 from all sites	94: non-small cell lung cancer, colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, prostatic carcinoma, hepatocellular carcinoma, ovarian carcinoma, breast carcinoma endometrial carcinoma, astrocytoma, melanomas, sarcoma, and bladder carcinoma	83.3	41.5
Marotta *et al.* (2012)	42 from all sites	120: prostate carcinoma, colorectal carcinoma, lung cancer (unspecified histology), hepatocellular carcinoma, gastric carcinoma, papillary thyroid carcinoma	Not defined (failed ROC analysis)	Not defined (failed ROC analysis)

GEP, gastroenteropancreatic; NEN, neuroendocrine neoplasm; ROC, receiving-operator characteristics.

cell carcinoma, lymphoma and metastatic lesions (Mulkeen *et al.* 2006). This is a challenging clinical issue as, despite the high specificity demonstrated by CT and MRI (Ichikawa *et al.* 2000, Sundin *et al.* 2009), the radiological phenotype of pancreatic NENs is variable, thus hampering instrumental diagnosis (Singhi *et al.* 2012). The retrospective analysis by Paik *et al.* (2013) reported only 56% specificity of the marker in differentiating pancreatic NENs from other pancreatic masses, whereas the prospective study by Jun *et al.* (2017), who specifically focused on patients suspected for a pancreatic NEN, showed a higher value, namely 77.8%, which even rose to 100% when selecting lesions larger than 4 cm. However, these data are not conclusive and the role of circulating CgA for the pre-surgical diagnosis of pancreatic NENs needs to be addressed in other clinical series.

Sensitivity

Sensitivity of circulating CgA can be considered as acceptable for functional and advanced NENs and extremely poor for localized non-functional disease. Since diagnosis of the latter is the most challenging for clinicians, this strongly limits the clinical utility of the test.

According to available studies (Table 1), sensitivity of circulating CgA for the diagnosis of NENs varies from 60 to 100% (Oberg 2011). This represents a significant range of variation, which does not allow to define test sensitivity as acceptable or not. Actually, this is due to the

fact that CgA levels are tightly related to disease-related features. Particularly, the main factors affecting the rate of abnormal CgA, which determines the sensitivity of the test, are tumor function and disease extent. Regarding the former, Janson *et al.* (1997) first reported a high rate of CgA elevation, namely 86.1%, in a large cohort of functional NENs from different sites. More recently, Nehar *et al.* (2004), focusing on a population of gastroenteropencreatic NENs, found CgA alterations in 70% of secreting tumors, whereas only 40% of the non-functional ones showed positivity for the CgA test. Regarding the role of disease extent, the Nehar study (Nehar *et al.* 2004) also found a significant difference in the rate of CgA elevation between metastatic and non-metastatic patients, namely 73 vs 26%. This dramatic impact on CgA sensitivity was further confirmed by Nikou *et al.* (2008), analyzing a cohort of non-functional pancreatic NEN, who found CgA alterations in the totality of patients with liver metastases, whereas the rate was much lower, 66.6%, in those subjects without liver involvement. According to these data, sensitivity of the CgA test can be considered as acceptable only for functional and advanced NENs. Recently, Jilesen *et al.* (2014), analyzing a cohort of non-metastatic non-functional pancreatic NENs, found CgA elevation in only 27% of the cases, further demonstrating the poor marker sensitivity in early non-functional disease. This strikingly limits clinical utility of circulating CgA as diagnosis of functional and metastatic NENs is mainly obtained by specific biomarkers and imaging modalities or biopsy, respectively (Jensen *et al.* 2012, Pavel *et al.* 2012),

whereas a stronger support from biochemistry would be specifically required for localized non-secreting NENs, where diagnosis is more challenging.

Diagnostic role of circulating CgA in particular clinical settings

CAG and inflammatory bowel diseases

CAG and inflammatory bowel diseases, already defined as non-oncological causes of CgA elevation, represent for clinicians a diagnostic challenge as they also predispose to NEN development through stimulating proliferation of neuroendocrine cells (Ruszniewski *et al.* 2006, West *et al.* 2007). The possible role of circulating CgA in discriminating patients with CAG and inflammatory bowel diseases who develop gastric (type I) and intestinal NEN, respectively, has been tested by few studies, with non-encouraging results. Peracchi *et al.* (2005) found higher CgA levels in CAG patients with gastric NEN, as compared with those without, but specificity of the test was extremely poor (23%), whereas Sciola *et al.* (2009) reported non-significant differences in CgA levels between inflammatory bowel diseases with and without concomitant intestinal NEN.

Multiple endocrine neoplasia type 1-related NENs

MEN1 is an autosomal dominant hereditary syndrome predisposing to the development of a variety of NENs. The most common are pancreatic NENs, occurring in 40–70% of the patients, whereas gastric, bronchial and thymic tumors are less frequent (Thakker *et al.* 2012). Importantly, NENs represent the main cause of MEN1-related death (Goudet *et al.* 2010). Therefore, a proper screening approach is required in order to detect NENs as early as possible, thus obtaining a reduction in MEN1-related morbidity and mortality (Pieterman *et al.* 2009, Ramundo *et al.* 2011). To date, the standard of care suggests annual CgA determination, but this statement is defined as 'low quality' (Thakker *et al.* 2012). Indeed, the actual value of circulating CgA as a screening test for NEN diagnosis in patients with MEN1 is still controversial as it has been assessed by very few studies. In 2003, Peracchi *et al.* (2003) found CgA alterations in the vast majority, 15 out of 16 cases, of MEN1 patients affected with gastroenteropancreatic NENs, and this was consistent with a possible use of CgA as the screening test. Nevertheless, 2 recent publications (de Laat *et al.* 2013, Qiu *et al.* 2016), analyzing larger cohorts of MEN1 subjects and specifically focusing on the detection of pancreatic NENs, provided opposite results. Indeed, both research

groups consistently demonstrated low diagnostic accuracy for CgA, also reporting poor sensitivity values, which is not feasible to use the marker as the screening test.

Circulating CgA as a prognostic marker in NEN

There is prospective evidence which demonstrates that advanced NENs secreting CgA have poorer outcome, as compared with those showing non-elevated marker levels. By contrast, data about localized disease are still poor.

Circulating CgA has long been used as an indirect survival predictor in clinical practice of NEN management. This was due to the well-demonstrated relationship with disease stage/extent, which represents the main predictor of clinical outcome (Ahmed *et al.* 2009). Indeed, the majority of authors demonstrated higher marker levels in patients with extensive metastases, as compared with those having localized disease or even limited hepatic involvement (Janson *et al.* 1997, Tomassetti *et al.* 2001*b*, Nehar *et al.* 2004, Campana *et al.* 2007, Zatelli *et al.* 2007, Nikou *et al.* 2008). Furthermore, Arnold *et al.* (2008) reported a direct correlation between the CgA increase and the extent of liver involvement. Nevertheless, such correlation is not valid for all NEN types as CgA levels may be affected by many other clinico-pathological features. As an example, due to direct tumor secretion and gastrin-induced ECL–cell hyperplasia, non-metastatic gastrinomas show CgA levels as high as those reported in metastatic non-functional pancreatic NENs (Janson *et al.* 1997), so the association with disease load and the indirect prognostic significance are lost in this case. Hence, the actual prognostic impact of circulating CgA can be assessed only analyzing the direct relationship with survival. This has been performed in a wide variety of retrospective analyses, all demonstrating that high CgA levels were predictors of poor survival (Janson *et al.* 1997, Arnold *et al.* 2008, Ekeblad *et al.* 2008, Nikou *et al.* 2008, Citterio *et al.* 2017, Nanno *et al.* 2017). Of note, the majority of these studies were focused on advanced disease, which has to be considered as the most important setting, since a proper prognostic stratification of these patients is mandatory. Arnold *et al.* (2008) showed that plasma CgA levels were related to survival time in a cohort of 344 patients with metastatic, well-differentiated NENs of gastroenteropancreatic origin. More recently, Citterio *et al.* (2017) evaluated a more homogeneous set of patients including 139 well-differentiated NENs with metastatic liver involvement. Authors identified basal CgA levels less than 200 ng/mL as a positive prognostic factor, and this

result was confirmed after a multivariate analysis. To date, prospective evidence about this issue has been provided by the RADIANT-1, -2 and -3 trials, where dedicated analyses were performed in order to assess a prognostic value of circulating CgA in the whole cohort of advanced NENs, independently of the prediction of treatment efficacy (Yao *et al.* 2008*b*, 2010, 2016, Pavel *et al.* 2017*b*). These studies considered patients showing circulating levels higher than 2-fold the upper normal limit as CgA secretors, while subjects with marker levels equal or below this cut-off were classified as having non-elevated CgA. In all cases, authors demonstrated shorter overall survival (OS) for patients with advanced NENs showing elevated CgA, who represented from 50 to 70% of the study cohorts. To date, data about localized disease are extremely poor. Recently, Nanno *et al.* (2017) analyzed a cohort of resectable, well-differentiated pancreatic NENs finding that preoperative serum CgA levels were significantly higher in patients with postoperative recurrence, as compared to those without recurrence. However, these findings need further confirmation in independent series.

Circulating CgA in the follow-up phase of NEN

The follow-up phase of NENs essentially includes 2 clinical situations: (a) patients being cured after surgery (R0 resection), where the objective is to identify relapses; (b) subjects with more advanced tumor who do not or cannot achieve a disease-free status, where the objective is to monitor morphological evolution, in order to detect transition from stable to progressive disease (Modlin *et al.* 2010*b*). Since these patients can be subjected to a variety of treatments, which are usually administered sequentially or even simultaneously during the course of the disease, monitoring the tumor slope represents the mainstay for a proper clinical management. Here, we report current evidence about the value of circulating CgA in each of the described settings.

Detection of tumor relapse after curative surgery

Evidence of the role of circulating CgA in this setting is still poor as based on few and controversial studies.

Initially, a retrospective study of 56 patients was consistent with a possible value of CgA as a marker of disease recurrence in midgut NENs subjected to radical surgery. Authors showed that CgA rising above the normal range represented the first indicator of the recurrence, even anticipating 5-hydroxyindolylacetic acid positivization

and instrumental examinations (Welin *et al.* 2009). Therefore, a twice-a-year CgA determination together with transabdominal ultrasonography was proposed as a feasible follow-up scheme. In contrast to these findings, a recent study providing prospective evaluation of 15 R0-resected gastroenteropancreatic NENs reported no CgA elevation in the 2 subjects developing recurrence who had elevated pre-surgical levels (Modlin *et al.* 2016). Despite the low number of cases, this was consistent with a poor utility of the marker in the follow-up of NENs after curative surgery.

Assessment of morphological evolution in patients with non-cured disease

Based on recently published prospective studies, the trend of circulating CgA over time cannot be considered as a valid marker of morphological evolution of NENs with persistent disease and its utility in the follow-up of these patients is therefore poor.

Initially, promising insights about the relationship between circulating CgA and morphological evolution of non-cured NENs were provided by 2 retrospective studies (Bajetta *et al.* 1999, Nehar *et al.* 2004). Both research groups found high concordance between CgA changes and tumor slope, demonstrating that marker elevation higher than 25% was a highly sensitive predictor (83 and 89%, respectively) of tumor progression. In contrast to these findings, a more recent retrospective study by Walter *et al.* (2012) found that marker changes were consistent with morphology in only 51% of the cases and that a significant CgA elevation, defined as an at least 50% increase, was detectable in only 56% of the patients with progressive disease. More definite evidence about this issue has emerged in the last 2 years, with 2 prospective reports demonstrating the poor capability of CgA changes in reflecting morphological behavior of NENs. In 2015, Cwikla *et al.* (2015) prospectively analyzed a cohort of 28 non-cured gastroenteropancreatic NENs treated with somatostatin analogues (SSA). Considering an at least 25% increase as cut-off, authors found low concordance, namely 64%, between CgA modifications and tumor slope. Particularly, only 57% of progressing patients showed significant CgA increase. Similarly, a 2017 study by Pavel *et al.* (2017*a*) of 34 advanced gastroenteropancreatic NENs subjected to various treatments, which also considered 25% increase as cut-off, reported only 40% concordance between morphological behavior and CgA modifications.

Circulating CgA for the definition of treatment strategy

Currently, a variety of tools are available for NEN treatment. Besides surgery, radical or debulking, these include several medical therapies (SSA, interferon alfa, targeted agents such as tyrosine kinase and mTOR inhibitors, and chemotherapy), PRRT and loco-regional treatments (radiofrequency ablation and transarterial embolization) (Modlin *et al*. 2010*b*, Oberg *et al*. 2010, Frilling *et al*. 2012, Pavel *et al*. 2012, Marotta *et al*. 2013, Del Prete *et al*. 2014, Fiore *et al*. 2014, Ramundo *et al*. 2014). This poses 2 major challenges for clinicians, who are required (a) to choose the best therapy for each specific patient; (b) to test as early as possible effectiveness of the chosen approach in order to perform a prompt adjustment of treatment strategy. Here, we analyze current role of circulating CgA in this context.

Baseline CgA as predictive marker of treatment efficacy

The best available evidence, deriving from two prospective placebo-controlled studies, shows no role of baseline CgA as a predictive marker of response to treatment.

Actually, promising insights about this issue were initially provided from a dedicated analysis extrapolated from the phase II RADIANT-1 study of everolimus in advanced pancreatic NETs (Yao *et al*. 2011*a*). Indeed, authors demonstrated that CgA levels >2 fold the upper normal limit were associated with significant reduction in both PFS and OS. However, the absence of a placebo group strongly limited the validity of the reported relationship, which could be simply due to the prognostic effect of CgA rather than to an actual interaction with the treatment. This thesis was subsequently confirmed by RADIANT-2 and -3, which were randomized, placebo-controlled trials of everolimus in advanced NENs with carcinoid syndrome under SSA treatment and advanced pancreatic NENs, respectively (Pavel *et al*. 2011, Yao *et al*. 2011*b*). Indeed, recent *post hoc* analyses of both studies specifically assessed whether baseline CgA levels were only prognostic or had actual capability of predicting treatment effect on OS (Yao *et al*. 2016, 2017, Pavel *et al*. 2017*b*). This was done by adjusting OS of the 2 study arms for pre-treatment CgA levels, which were imbalanced. Authors concluded that baseline CgA was not predictive of everolimus impact on outcome. Recently, the lack of predictive value of pre-treatment marker levels was also suggested for NENs subjected to PRRT, an established

therapeutic modality mainly used for inoperable or metastatic gastroenteropancreatic NENs (van der Zwan *et al*. 2015). Even if limited by the lack of a control group of untreated subjects, a recent prospective study by Bodei *et al*. (2016) showed no impact of elevated CgA (>600 ng/mL) on both morphological response and PFS.

CgA response as a predictive marker of treatment efficacy

Subanalyses from 2 prospective placebo-controlled studies reported CgA response as a predictor of medical treatment efficacy. Regarding surgery and PRRT, the best prospective evidence, based on single dedicated studies, shows no value of CgA changes as a predictive marker of response.

The mentioned analysis from the RADIANT-1 reported that, among patients with elevated baseline levels, an early CgA response, defined as an at least 30% reduction of the marker at 4 weeks treatment, was predictive of morphological response, PFS, and OS (Yao *et al*. 2011*a*). This was consistent with the previous retrospective observation that an early CgA decrease was associated with improved RECIST response and clinical outcome in pancreatic NENs subjected to streptozocin-based chemotherapy (Kouvaraki *et al*. 2004). As previously discussed, these findings were intrinsically limited by the absence of a control group. However, the role of CgA reduction as a predictive marker of response to medical therapies has found some confirmation through dedicated subanalyses of placebo-controlled trials. In 2011, a contribution to the European Society for Medical Oncology Congress, based on data from the RADIANT-2 trial, confirmed that early CgA responders had longer PFS, as compared with non-responders (Baudin *et al*. 2011). More recently, a subanalysis of the CLARINET study, a randomized phase III trial of lanreotide in advanced NENs, showed that a decrease in CgA was associated with reduced hazard of disease progression (Buil-Bruna *et al*. 2016). Regarding surgery and PRRT, the mentioned Modlin study (Modlin *et al*. 2016), providing prospective evaluation of gastroenteropancreatic NENs treated with surgery, found no significant postsurgical CgA changes between cured and non-cured patients. Similarly, the prospective Bodei study (Bodei *et al*. 2016) about PRRT found that the rate of CgA reduction was higher in non-responders than in responding cases (21 and 40%, respectively), thus indicating poor utility of CgA modifications in predicting treatment efficacy.

Conclusions

A wide body of research has been dedicated over the last 2 decades to define clinical application of circulating CgA in NENs. As all authors agree, the marker is intrinsically limited by the lack of assay standardization generating significant variations across different laboratories. This depends not only on the applied technique, but also on the employed antibody when using the same method (Modlin *et al.* 2010*a*), and hampers not only management of a single patient, but also the comparison between different studies, thus making hard to define the actual marker performance. Clinical value of CgA in the diagnostic setting is hampered by issues impairing both specificity and sensitivity. Regarding the former, the major problem is that several conditions other than NEN can affect CgA levels, therefore acting as confounding factors. These include some highly prevalent non-oncological conditions, such as gastrointestinal and cardiovascular disorders or PPIs assumption, and a variety of non-NEN tumors. Among the latter, those with the highest impact in clinical practice are malignancies arising from anatomic areas where NENs occur more frequently, such as colorectal and pancreatic adenocarcinoma. Sensitivity of the test is intrinsically limited by the fact that a relevant portion of NENs, 30–50%, do not show elevated CgA levels (Lindholm & Oberg 2011). Due to the tight correlation of the marker with tumor function and disease extent, this issue mainly involves NENs with non-functionally localized disease where CgA is normal in about 70% of the cases (Jilesen *et al.* 2014). In this kind of patients, where the role of clinics and instrumental exams is limited and the need of an accurate biochemical marker is higher, a diagnostic role of circulating CgA is paradoxically marginal, due to the poor sensitivity. The insufficient diagnostic utility of CgA has been recently formalized in a Delphi consensus, stating that no single circulating biomarker meets the minimum required standard, namely sensitivity and specificity higher than 80 and 90%, respectively, to be considered as a supportable diagnostic tool (Oberg *et al.* 2015). This contrasts with current indications from the ENETS and the other major societies dealing with NENs, which still recommend broad spectrum use of circulating CgA for the diagnostic definition (Kloppel *et al.* 2009, O'Toole *et al.* 2009, Jensen *et al.* 2012, Oberg *et al.* 2012*a*,*b*, Ramage *et al.* 2012, Kunz *et al.* 2013, Caplin *et al.* 2015, Kulke *et al.* 2015, Falconi *et al.* 2016, Niederle *et al.* 2016). It is our opinion that societal guidelines should be updated better addressing the actual diagnostic value of circulating CgA, thus avoiding incorrect and expensive use of the marker.

Regarding the prognostic value of CgA, there is prospective evidence that advanced NENs with elevated CgA have poor outcome. Owing to the wide variability in disease evolution of stage IV NENs (Baudin 2007, Baudin *et al.* 2012), which makes essential to perform prognostic stratification of these patients, this can be considered as the most important clinical application of circulating CgA. However, elevated CgA is detectable in a wide proportion, namely 50–70%, of advanced NENs, so the identification of cut-offs allowing a proper risk stratification among patients secreting the protein is required.

Regarding the follow-up phase, recent prospective studies (Cwikla *et al.* 2015, Pavel *et al.* 2017*a*) show that circulating CgA does not represent a valid marker of morphological evolution of disease and has therefore no utility in this setting.

Regarding the role of CgA for the definition of treatment strategy, available evidence is overall poor as limited by the small number of dedicated studies and, also, by the retrospective nature as well as the absence of control groups of untreated subjects characterizing the majority of them. Nevertheless, dedicated analyses from the most recent prospective placebo-controlled trials (Yao *et al.* 2016, 2017, Pavel *et al.* 2017*b*) demonstrated no CgA role for predicting the impact of a medical treatment on survival. However, it is important to remark that CgA, per definition, can be used as a marker only in NENs showing abnormal serum level. Since the portion of NENs with normal CgA is remarkable, accounting for 30–50% of the patients, this strongly limits the actual clinical application of the marker.

In conclusion, despite representing the best available monoanalyte marker related to NEN (Modlin *et al.* 2010*b*, Kulke *et al.* 2015), CgA carries the typical limitations of single-analyte measurements (Hood & Tian 2012), and is therefore unable to provide comprehensive evaluation of a heterogeneous entity such as NEN (Baudin 2007, Yao *et al.* 2008*a*). Hence, the new frontier seems to be represented by multianalyte approaches. Particularly, a blood-based algorithm including simultaneous determination of 51 NEN-specific markers has been developed in recent years (Modlin *et al.* 2013), and all comparative studies were concordant in reporting significantly better metrics, as compared with CgA (Modlin *et al.* 2014*a*,*b*, 2015, 2016, Bodei *et al.* 2016, Pavel *et al.* 2017*a*).

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