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

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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.

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, enolase, and specific or individual, including serotonin 5-HT2c receptor, somatostatin, 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 (hCgA 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
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. 1992a, 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 ($−20^\circ$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>
<thead>
<tr>
<th>Study (year)</th>
<th>N</th>
<th>Primary location</th>
<th>Stage IV (N)</th>
<th>Comparison group</th>
<th>Interfering diseases</th>
<th>PPIs assumption</th>
<th>Assay</th>
<th>Cut off values</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomassetti et al. (2001a,b)</td>
<td>80</td>
<td>GEP</td>
<td>NA</td>
<td>28 HS</td>
<td>Excluded</td>
<td>Not considered as exclusion criterion</td>
<td>ELISA</td>
<td>17 U/L</td>
<td>56</td>
<td>100</td>
</tr>
<tr>
<td>Bernini et al. (2001)</td>
<td>48</td>
<td>All sites</td>
<td>NA</td>
<td>130 HS</td>
<td>Excluded</td>
<td>Not considered as exclusion criterion</td>
<td>RIA</td>
<td>100 ng/mL</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Peracchi et al. (2003)</td>
<td>61</td>
<td>GEP</td>
<td>28</td>
<td>50 HS</td>
<td>Not considered as exclusion criteria</td>
<td>Not considered as exclusion criterion</td>
<td>ELISA</td>
<td>20 U/L</td>
<td>92</td>
<td>83</td>
</tr>
<tr>
<td>Zatelli et al. (2004)</td>
<td>124</td>
<td>GEP</td>
<td>97</td>
<td>50 HS</td>
<td>Excluded</td>
<td>Not considered as exclusion criterion</td>
<td>IRMA</td>
<td>100 μg/L</td>
<td>66</td>
<td>100</td>
</tr>
<tr>
<td>Zatelli et al. (2007)</td>
<td>81</td>
<td>GEP</td>
<td>NA</td>
<td>129 HS</td>
<td>Excluded</td>
<td>Not considered as exclusion criterion</td>
<td>IRMA</td>
<td>53 ng/mL</td>
<td>71.3</td>
<td>84</td>
</tr>
<tr>
<td>Campana et al. (2007)</td>
<td>238</td>
<td>GEP, lung</td>
<td>95</td>
<td>48 HS</td>
<td>Excluded</td>
<td>Not considered as exclusion criterion</td>
<td>ELISA</td>
<td>18 U/L</td>
<td>85.3</td>
<td>95.8</td>
</tr>
<tr>
<td>Belli et al. (2009)</td>
<td>119</td>
<td>GEP</td>
<td>84</td>
<td>39 HS</td>
<td>Excluded</td>
<td>Not considered as exclusion criterion</td>
<td>RIA</td>
<td>2.8 nmol/L</td>
<td>92.3</td>
<td>100</td>
</tr>
<tr>
<td>Molina et al. (2011)</td>
<td>66</td>
<td>All sites</td>
<td>45</td>
<td>52 HS</td>
<td>Excluded</td>
<td>Not considered as exclusion criterion</td>
<td>IRMA</td>
<td>90 ng/mL*</td>
<td>80.3</td>
<td>100</td>
</tr>
<tr>
<td>Vezzosi et al. (2011)</td>
<td>184</td>
<td>GEP</td>
<td>44</td>
<td>No comparison</td>
<td>Not considered as exclusion criterion</td>
<td>Not considered as exclusion criterion</td>
<td>ELISA</td>
<td>60 ng/mL*</td>
<td>83.3</td>
<td>100</td>
</tr>
<tr>
<td>Marotta et al. (2012)</td>
<td>42</td>
<td>All sites</td>
<td>8</td>
<td>100 HS (benign</td>
<td>Not considered as exclusion criterion</td>
<td>Not identified</td>
<td>Not defined</td>
<td>Not defined</td>
<td>Not defined</td>
<td></td>
</tr>
<tr>
<td>Korse et al. (2012)</td>
<td>573**</td>
<td>All sites</td>
<td>388</td>
<td>282 HS</td>
<td>Not considered as exclusion criterion</td>
<td>Not considered as exclusion criterion</td>
<td>RIA</td>
<td>87 μg/L</td>
<td>69</td>
<td>95</td>
</tr>
<tr>
<td>Modlin et al. (2013)</td>
<td>81</td>
<td>GEP</td>
<td>NA</td>
<td>94 HS</td>
<td>Not considered as exclusion criterion</td>
<td>Not considered as exclusion criterion</td>
<td>ELISA</td>
<td>19 U/L</td>
<td>32</td>
<td>99</td>
</tr>
<tr>
<td>Tohmola et al. (2014)</td>
<td>41</td>
<td>All sites</td>
<td>NA</td>
<td>26 HS</td>
<td>Not considered as exclusion criterion</td>
<td>Not considered as exclusion criterion</td>
<td>RIA</td>
<td>6 nmol/L</td>
<td>51</td>
<td>86</td>
</tr>
</tbody>
</table>

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. 2001a, 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. 2001b, 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. 2001b, 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. 2010a).

**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.**

<table>
<thead>
<tr>
<th>Non-oncological</th>
<th>Iatrogenic causes</th>
<th>Oncological</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Benign diseases</strong></td>
<td>Proton pump inhibitors</td>
<td>Colorectal carcinoma</td>
</tr>
<tr>
<td>Gastrointestinal: chronic atrophic gastritis, Helicobacter pylori infection, liver cirrhosis, chronic hepatitis, pancreatitis, inflammatory bowel diseases, irritable bowel</td>
<td>Histamine 2 receptor antagonists</td>
<td>Gastric carcinoma</td>
</tr>
<tr>
<td>Cardiovascular: hypertension, heart failure, acute coronary syndromes</td>
<td>Serotonin reuptake inhibitors</td>
<td>Pancreatic carcinoma</td>
</tr>
<tr>
<td>Renal and hepatic dysfunctions</td>
<td></td>
<td>Prostate carcinoma</td>
</tr>
<tr>
<td>Others: giant cell arteritis, rheumatoid arthritis, systemic lupus erythematosus, pulmonary obstructive disease, hyperthyroidism</td>
<td></td>
<td>Breast carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian carcinoma</td>
</tr>
</tbody>
</table>
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 NYHA 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. 2009a;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 compensative hypergastrinemia and G-cell hyperplasia, which in turn induce ECL-cell hyperplasia. Both G- and ECL-hyperplasia are responsible for CgA overproduction (Kuijpers 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 inducers 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. 2001b, 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 ducal 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.

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

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 gastroenteropancreatic 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. 2001b, 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. 2008b, 2010, 2016, Pavel et al. 2017b). 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. 2010b). 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. (2017a) 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. 2010b, Obereg 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. 2011a). 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. 2011b). 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. 2017b). 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 (>600ng/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. 2011a). 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. 2010a), 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, Obeg et al. 2012a,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. 2017a) 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. 2017b) 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. 2010b, 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. 2008a). 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. 2014a,b, 2015, 2016, Bodei et al. 2016, Pavel et al. 2017a).
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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