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INVITED REVIEW

Circulating chromogranin A and its fragments as diagnostic and prognostic disease markers

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Abstract Chromogranin A (CgA), a secretory protein released in the blood by neuroendocrine cells and neurons, is the precursor of various bioactive fragments involved in the regulation of the cardiovascular system, metabolism, innate immunity, angiogenesis, and tissue repair. After the original demonstration that circulating CgA can serve as a biomarker for a wide range of neuroendocrine tumors, several studies have shown that increased levels of CgA can be present also in the blood of patients with cardiovascular, gastrointestinal, and inflammatory diseases with, in certain cases, important diagnostic and prognostic implications. Considering the high structural and functional heterogeneity of the CgA system, comprising precursor and fragments, it is not surprising that the different immunoassays used in these studies led, in some cases, to discrepant results. Here, we review these notions and we discuss the importance of measuring total-CgA, full-length CgA, specific fragments, and their relative levels for a more thorough assessment of the pathophysiological function and diagnostic/prognostic value of the CgA system.

Keywords Chromogranin A · Vasostatins · Catestatin · Serpinin · Neuroendocrine tumors · Cardiovascular diseases · Inflammatory diseases

Abbreviations

CgA	chromogranin A
VIF	vasoconstrictive-inhibitory factor
ICAM-1	intercellular adhesion molecule-1
MCP-1	monocyte chemoattractant protein-1
HMGB-1	high mobility group box-1
TNF	tumor necrosis factor alpha
IFN	interferon
VEGF	vascular endothelial growth factor
FGF2	fibroblast growth factor-2
BNP	brain natriuretic peptide
PPIs	proton pump inhibitors
ELISA	enzyme-linked immunosorbent assay

Introduction

Chromogranin A (CgA) is an acidic glycoprotein originally discovered as the major soluble protein of adrenal medullary chromaffin granules [5]. This protein was later found to be a major member of a family of regulated secretory proteins, collectively called "granins", present in the electron-dense granules of many normal and neoplastic neuroendocrine tissues and of the nervous system [26, 46, 51, 81, 94]. Upon exocytosis, CgA is released together with the co-stored hormones in the extracellular environment and then in the circulation. Intra-granular and/or extracellular proteolysis of CgA can generate a variety of bioactive fragments involved in the regulation of the cardiovascular system, metabolism, innate immunity, angiogenesis, and tissue repair [4, 45, 46, 58, 94,

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98]. O'Connor and Bernstein were the first to demonstrate that human circulating CgA can serve as a biomarker for a wide range of neuroendocrine tumors [68]. Later studies showed that increased levels of CgA are present also in the blood of patients with cardiovascular, gastrointestinal, and inflammatory diseases, in certain cases with important diagnostic and prognostic implications [46, 58]. Most of these studies were performed by measuring serum or plasma CgA immunoreactivity using various homemade or commercial immunoassays based on different antibodies. Given the complexity of the CgA system, which consists of full-length molecules and various fragments, it comes as no surprise that the use of assays detecting different CgA epitopes led to discrepant results or, in any case, to a system description very far from being complete. The purpose of this article is to review and discuss these notions and to provide our point of view regarding the importance of measuring not only total-CgA immunoreactivity, but also full-length CgA, specific fragments and their relative levels, for a more thorough assessment of the pathophysiological function of circulating CgA and of its diagnostic/prognostic value.

Chromogranin A and its fragments: structure and function

Human CgA is a 439-residue-long protein expressed by neuroendocrine cells of the gastrointestinal and respiratory tracts, adeno- and neuro-hypophysis, parathyroid glands, endocrine pancreas, thyroid, and also by atrial and ventricular cardiomyocytes, wound keratinocytes, germinal epithelial cells, human polymorphonuclear neutrophils, and lymphoid organs [46]. In different tissues, CgA can undergo differential post-translational modifications, including glycosylation, sulfation, phosphorylation, and proteolytic cleavage [36, 46, 88]. Proteolytic processing of CgA can be triggered by intragranular and/or extracellular proteases, such as prohormone convertase 1 and 2, furin, cathepsin L, plasmin, and thrombin [8–10, 21, 23, 30, 31]. Experimental evidence obtained in various in vitro and in vivo models suggests that CgA and its fragments, after secretion, can exert various regulatory functions. These fragments and their biological effects have been extensively reviewed [4, 45, 46, 58, 94, 98, 100]. Here, we will briefly describe only the most intensively investigated CgA-related polypeptides that might have a regulatory role in pathophysiological conditions and, consequently, a potential value as diagnostic/prognostic disease markers (see Fig. 1 for a schematic representation). These polypeptides include full-length CgA, large fragments lacking the C-terminal region, and shorter fragments, like $CgA_{1–76}$ (called vasostatin-I), $CgA_{1–113}$ (vasostatin-II), $CgA_{79–113}$ (vasoconstrictive-inhibitory factor, VIF), $CgA_{250–301}$ (pancreastatin), $CgA_{352–372}$ (catestatin), and $CgA_{411–436}$ (serpinin). For example, experimental evidence has shown that vasostatin-I and vasostatin-II, i.e. fragments corresponding to the N-terminal region, exert vasodilatory effects [1, 2, 46]. Also, VIF is a vasoregulatory molecule that modulates the vasoconstrictive activity of angiotensin II, by acting on the angiotensin II type 2 receptor [86]. Vasostatin-I inhibits parathyroid hormone secretion, exerts antibacterial/antifungal activity, and induces neurotoxic effects in neuronal/microglial cell cultures [46, 90, 100]. Pancreastatin inhibits insulin secretion and regulates glucose and lipid metabolism [37, 69, 87, 108], whereas catestatin inhibits nicotinic cholinergic-stimulated catecholamine secretion, induces vasodilation and monocyte chemotaxis, stimulates rat mast cells to release histamine, and acts as an antimicrobial and antimalarial peptide [62, 90]. Serpinin, a fragment corresponding to the C-terminal region of CgA, exerts protective effects against oxidative stress on neurons and pituitary cells [58]. CgA and its fragments also work as cardio-regulatory hormones, in certain cases with opposite functions. In particular, full-length CgA, vasostatin-I, and catestatin depress myocardial contractility and relaxation, counteract the β-adrenergic-induced positive inotropism, and modulate the coronary tone, principally via a nitric oxide-dependent mechanism [98]. Conversely, the fragment serpinin enhances myocardial contractility and relaxation, via a β-adrenergic-cAMP/protein kinase A and nitric oxide-independent pathway [76, 99]. Vasostatin-II, a fragment that contains the vasostatin-I sequence, increased coronary pressure in Langendorff-perfused rat hearts without affecting inotropism but counteracted the cardio-stimulatory effects of isoproterenol [17]. CgA and vasostatin-I can also modulate, in an opposite manner, the adhesion of fibroblasts, smooth muscle cells, cardiomyocytes, and of endothelial cells to proteins of the extracellular matrix [46]. Interestingly, CgA and its N-terminal fragments can interact with αvβ6 integrin on keratinocytes, through an Arg-Gly-Asp (RGD) site located within residues 43–45, and promote wound healing in a murine model [25].

Physiological concentrations of CgA and vasostatin-I preserve the integrity of the endothelial barrier and inhibit tumor necrosis factor alpha (TNF)-induced vascular leakage by preventing the disassembly of vascular endothelial (VE)-cadherin-dependent adherence junctions [7, 11, 35]. Vasostatin-I inhibits TNF-induced intercellular adhesion molecule-1 (ICAM-1) expression, monocyte chemoattractant protein-1 (MCP-1) release, and relocation of high-mobility group box-1 (HMGB-1) in human microvascular endothelial cells, suggesting an anti-inflammatory role for this fragment [28]. Vasostatin-I inhibits also the pro-permeability effects of TNF and interferon (IFN)-γ on intestinal epithelial cell monolayers, suppresses lipopolysaccharide-induced production of the inflammatory chemokine KC (an interleukin 8 homolog), and protects mice from dextran sulfate sodium-induced colitis when administered orally [85].

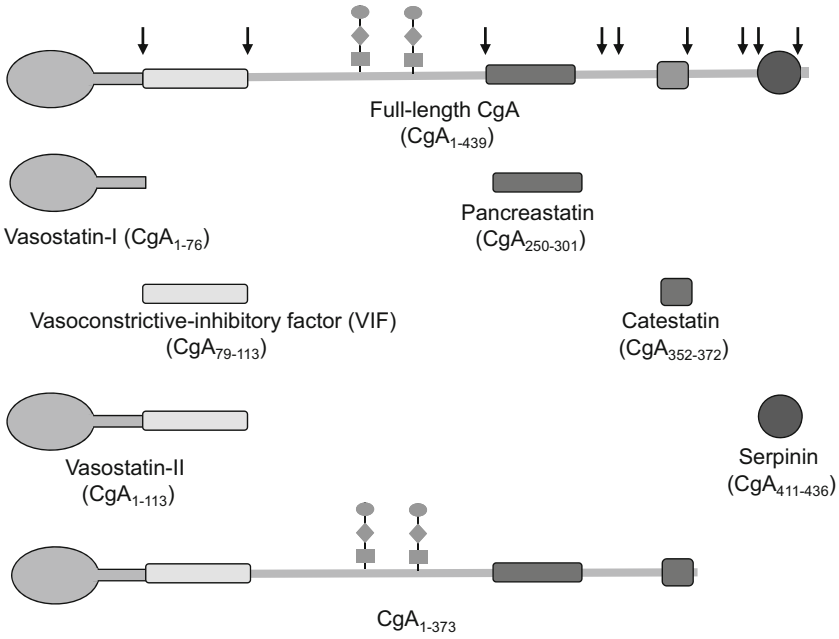

Fig. 1 Schematic representation of full-length chromogranin A ($CgA_{1–439}$) and of the most intensely investigated bioactive fragments that have a potential value as diagnostic/prognostic disease markers. *Arrows* indicates dibasic sites of potential cleavage. Glycosylation sites are also indicated

A growing body of evidence suggests that physiological concentrations of CgA and some of its fragments may also have a regulatory role in angiogenesis. For example, full-length CgA can inhibit angiogenesis in the chick embryo chorioallantoic membrane model [23]. Furthermore, $CgA_{1–439}$ and vasostatin-I at 0.1–1 nM concentrations can inhibit capillary sprouting induced by vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2) from rat aortic rings cultured in 3D-collagen gels [23]. Both polypeptides can also inhibit in vivo angiogenesis and tumor growth in various models of solid tumors [24]. Functional anti-angiogenic sites are located in the C- and N-terminal regions (residues 410–439 and 1–76), the latter requiring cleavage for full activation [23]. According to this view, systemic administration of $CgA_{1–78}$ could prevent choroidal neovascularization and vascular leakage in an established mouse model of laser-induced ocular neovascularization [60]. Mechanistic studies have shown that the anti-angiogenic and anti-tumor activities of the full-length CgA require the induction of protease-nexin 1 in endothelial cells, a serine protease inhibitor endowed with anti-angiogenic activity [24].

Interestingly, while full-length $CgA_{1–439}$ inhibits FGF2-induced angiogenesis, the fragments $CgA_{1–373}$ and $CgA_{352–372}$ (catestatin) can induce the release of FGF2 from endothelial cells and exert pro-angiogenic effects [23, 96]. Furthermore, $CgA_{1–439}$, $CgA_{1–76}$, and $CgA_{1–373}$ can counterbalance the pro-/anti-angiogenic activity of each other [8], suggesting that these polypeptides may form a balance of anti- and pro-angiogenic factors. Limited digestion with thrombin and plasmin can cleave the R_{373}–R_{374} dibasic site of CgA and tip the balance toward a pro-angiogenic state in pathophysiological conditions characterized by enzyme activation, e.g. in wound healing and cancer [8, 23]. However, extensive digestion of CgA with thrombin or plasmin may generate more fragments with unknown functions [8].

Although further work is necessary to elucidate the receptor mechanisms underlying the various biological effects exerted by CgA and its fragments, the knowledge accumulated so far suggests that the presence of these molecules in circulation is not just an epiphenomenon of cell secretory activity, but that they may have a physiological function. Thus, changes in their circulating levels, as observed in certain diseases (see Table 1, discussed below) might have a pathophysiological role. At this regard, it is also important to mention that the dose-response curves of CgA and its fragments in several biological assays, e.g. in angiogenesis, cell adhesion, and heart contractility assays, are bell-shaped [23, 76]. Consequently, an excessive increase in their circulating levels may paradoxically lead to lower biological effects, for unknown reasons. Thus, accurate qualitative and quantitative characterization of CgA and fragments in normal subjects and patients is necessary to fully assess the biological function of the CgA system, and its potential diagnostic and prognostic values.

Circulating CgA in normal subjects

Several radio- and enzyme-immunoassays based on antibodies against different epitopes of CgA have been employed to quantify the levels of this protein in the blood and other body fluids of normal subjects. Given the highly heterogeneous nature of the CgA antigen, owing to the differential post-

Table 1 Extracellular biological activities of CgA and its fragments and pathological conditions associated with changes in their circulation levels compared to normal subjects (see text for references)

CgA-related polypeptide	Biological functions	Pathological conditions associated with changes (positive or negative) in the circulation levels
CgA[a] (undefined structure)	ND[b]	Neuroendocrine tumors, tumors with neuroendocrine differentiation, chronic heart failure, myocardial infarction, renal failure, hypertension, sepsis, arteritis, Erdheim-Chester disease, rheumatoid arthritis, atrophic gastritis, inflammatory bowel disease, Meniere's disease
$CgA_{1–439}$ (full-length CgA)	Angiogenesis (↓), myocardial contractility and relaxation (↓), cell adhesion (↓), endothelial barrier (↑), wound healing (↑), tumor growth (↓)	Multiple myeloma, carotid artery atherosclerosis
$CgA_{1–76}$ (vasostatin-I)	Antibacterial, neurotoxic, antifungal, vasodilation (↑), angiogenesis (↓), myocardial contractility (↓), cardioprotection (↑), parathyroid hormone release (↓), cell adhesion (↑), endothelial barrier (↑), vascular leakage (↓), tumor growth (↓), gastrointestinal pain (↓), intestinal epithelial cell permeability (↓)	Multiple myeloma, carotid artery atherosclerosis, sepsis, Takayasu's arteritis
$CgA_{1–113}$ (vasostatin-II)	Heart remodeling and fibrosis (↓), ischemic cardioprotection (↑)	Ischemic chronic heart failure, coronary artery atherosclerosis
$CgA_{79–113}$ (VIF)	Vasodilation (↑)	Heart failure, kidney failure
$CgA_{1–373}$	Angiogenesis (↑)	Multiple myeloma
$CgA_{250–301}$ (pancreastatin)	Insulin secretion (↓), regulates glucose and lipid metabolism	Gestational diabetes
$CgA_{352–372}$ (catestatin)	Myocardial contractility and relaxation (↓), cardioprotection (↑), angiogenesis (↑), catecholamine secretion (↓), vasodilation (↑), monocyte chemotaxis (↑), histamine release from mast cells (↑), antimicrobial and antimalarial	Chronic heart failure, myocardial infarction, malignant arrhythmia, acute coronary syndrome, unstable angina
$CgA_{411–436}$ (serpinin)	Myocardial contractility and relaxation (↑), cardioprotection (↑), oxidative stress (↓), protease-nexin-1 (↑)	ND[b]

[a] The term "CgA (undefined structure)" refers to CgA molecules typically detected with immunoassays that cannot distinguish precursors from fragments

[b] *ND* not determined

translational modifications and proteolytic processing, and considering that a recognized international standard was not available for these studies, it is not surprising that a wide range of average concentrations (from 0.5 to 5 nM) has been reported for the normal circulating levels of CgA [16, 42, 46, 91, 93]. Using a sandwich ELISA based on a monoclonal antibody against the vasostatin-I region in the capture step and antibodies against the CgA central region in the detection step (thus unable to discriminate between full-length CgA and fragments lacking the C-terminal region), we observed that this protein circulates at about 0.5–1 nM [23]. Using assays based on antibodies specific for full-length CgA or for certain fragments, we observed that the circulating pool in normal subjects is highly heterogeneous, consisting of full-length CgA (about 0.1 nM) and a larger proportion of fragments lacking part of, or the entire, C-terminal region [23]. In addition, normal plasma contains vasostatin-I (about 0.3–0.4 nM). Considering that the circulating levels of $CgA_{1–439}$ and vasostatin-I in normal subjects are biologically relevant in terms of angiogenesis inhibition, both molecules might contribute to the homeostatic inhibition of angiogenesis in normal conditions. Other fragments not detected by these assays might also be present. For example, catestatin has been reported to be present in circulation at concentrations ranging from 0.03 to 1.5 nM in normal subjects [53, 65, 71]. The molecular entities containing the catestatin sequence detected by these studies were not characterized. Thus, the discrepancies between these values might be related again to the use of different antibodies capable of detecting catestatin as well as larger precursors with different efficiency.

CgA in patients with neoplastic diseases

Neuroendocrine tumors

CgA is expressed by many endocrine and neuroendocrine tumors, including pheochromocytomas, various carcinoid

tumors of the stomach, intestine, and lung, medullary thyroid carcinoma, parathyroid carcinoma, anterior pituitary tumors, neural tumors, pancreaticoduodenal tumors, small cell lung cancer, and many others [26, 46, 51, 81, 94]. In addition, certain tumors, such as prostate cancer, non-small cell lung cancer, breast cancer, and gastric and colonic adenocarcinomas, may undergo neuroendocrine differentiation and present focal expression of CgA. Thus, neuroendocrine tumors are composed, at least in part, of specialized cells that have the ability to produce, store, and secrete CgA, along with bioactive amines and other peptide hormones. Secreted CgA may then enter the bloodstream, thereby achieving levels higher than in normal subjects and acquiring the potential to act as a biomarker of the underlying neoplasm. Therefore, measurement of CgA levels in serum or plasma may reflect the presence of neuroendocrine tumors or tumors with neuroendocrine differentiation.

While increased levels of CgA may reflect the presence of an underlying neuroendocrine tumor, the question arises as to when the determination of CgA levels is useful for this purpose. At this regard it is important to keep in mind that neuroendocrine tumors can release, in addition to CgA, also other hormones and bioactive molecules, which may be more typical of a given neuroendocrine tumor. However, in some cases, neuroendocrine tumors do not produce and release bioactive molecules other than CgA. Thus, in these cases, referred to as "non-functioning neuroendocrine tumors", CgA may represent the only available biomarker. The measurement of CgA levels may be useful also when other bioactive molecules released by a given neuroendocrine tumor are unstable, rapidly fluctuate or require laborious assays.

While elevated levels of CgA in the bloodstream may be a marker of an underlying neuroendocrine tumor, it should be taken into consideration that also certain non-neoplastic diseases may lead to enhanced production and release of CgA. Benign conditions that can be accompanied by elevated CgA levels in the bloodstream include neuroendocrine hyperplasia, essential hypertension, organ failure, inflammatory or autoimmune conditions (like rheumatoid arthritis), primary hyperparathyroidism, chronic atrophic gastritis, and certain cardiovascular diseases that will be described below [38]. Moreover, administration of proton pump inhibitors, which are among the most commonly prescribed drugs, may cause a secondary increase in CgA levels due to increased gastrin production [88]. The fact that also non-neoplastic conditions can cause an increase of CgA levels explains why certain subjects may have highly variable levels of CgA that can overlap with those observed in patients with neuroendocrine tumors [44, 106]. While an all-or-nothing criterion cannot be applied for CgA in order to discriminate between the presence and absence of a neuroendocrine tumor, CgA levels in certain patients can reach levels that are not observed in other diseases. Thus, tumors like metastatic carcinoid tumors can display levels of serum CgA that are up to 1000 times the upper limit of the normal range [70]. This is approximately one–two orders of magnitude more than what is generally observed in non-malignant diseases with elevated CgA levels. Also in this case, however, there are exceptions. For example, CgA levels in patients with end-stage renal disease can attain very high levels, suggesting that kidneys have an important role in eliminating CgA [47].

Given these considerations, the usefulness of CgA as a biomarker of neuroendocrine tumors rests on an upfront differential diagnosis that allows excluding the presence of confounding non-malignant conditions that may cause elevation of CgA levels. Very high levels of CgA are almost pathognomonic of certain neuroendocrine tumors, but also in this case, it seems appropriate to exclude some pathological conditions that may be causative of high CgA levels (e.g. renal insufficiency). Once it has been established that increased levels of CgA in the bloodstream are due to the presence of a neuroendocrine tumor, CgA may be a useful marker to follow the tumor burden and response to treatment, as well as the clinical course of the disease and prediction of survival [94]. Thus, in multiple endocrine neoplasia type I, a clear correlation between the tumor mass and the circulating level of CgA has been found [41]. In patients with midgut carcinoid tumors, elevated CgA was an independent predictor of death [33]. In small-cell lung cancer, the plasma level of CgA reflected response to treatment and was useful in monitoring patients for recurrent disease [94]. In prostate cancer, high levels of serum CgA are a marker of advanced disease, associated both with tumor grade and disease stage [12]. Serum CgA may also predict prognosis in castration-resistant prostate cancer following endocrine therapy [6, 49] and can be used to distinguish between malignant and benign pheochromocytomas, with the malignant neoplasms attaining much higher levels than their benignant counterparts [81]. Higher CgA levels are generally observed in metastatic patients compared with those without metastases. In patients with very extensive metastatic spread, however, CgA levels can be lower [106]. This might be the consequence of a possible loss of neuroendocrine differentiation, probably reflecting a more aggressive behavior. In fact, CgA is normally absent or only focally expressed in poorly differentiated endocrine carcinomas. On the other hand, neuroendocrine differentiation in prostate tumors is associated with poorer prognosis [105], and large-cell carcinomas of the lung with neuroendocrine features are more clinically aggressive than classic large-cell carcinomas [50]. However, it is very difficult to speculate on whether CgA might exert detrimental effects in patients based on these associations. Experiments in animal models have shown that tumor cells genetically engineered to secrete full-length CgA are less aggressive that non-secreting cells [20]. Furthermore, administration of low-dose full-length CgA can inhibit tumor growth in various animal models of solid tumors [7, 24],

which apparently argue in favor of an anti-tumor activity of CgA. However, it is important to keep in mind that the overall biological effects of CgA can depend on its local concentration, its proteolytic processing, and its post-translational modifications, which may vary from tumor to tumor and even in different tumor areas. It is possible, therefore, that the unbalanced production of CgA polypeptides (e.g. pro-/anti-angiogenic) owing to excessive proteolytic processing in certain tumors or tumor areas might actually contribute to sustain angiogenesis and growth. Thus, detailed characterization of all local and circulating forms of CgA in cancer patients is necessary to elucidate the pathophysiological role of the CgA system.

In summary, and as a general rule, the diagnostic usefulness of CgA as a marker appears to be higher for well than for poorly differentiated tumors and for metastatic than for locoregional neoplasms. Moreover, CgA is more reliable for the evaluation of response to therapy or disease progression than for early diagnosis or recurrence. However, it is also important to highlight the fact that for certain tumors, e.g. pancreatic neuroendocrine tumors, the utility of circulating CgA for tumor diagnosis/prognosis is very limited, considering the various confounding factors discussed above. Given that the proteolytic machinery is typically altered in tumors, the plasma levels of specific CgA fragments might represent more useful and reliable disease biomarkers [91], a possibility that merits further investigation.

Non-neuroendocrine tumors

Plasma CgA is increased in a subpopulation of patients with non-small cell lung cancer, independently of neuroendocrine differentiation, and correlates with worsening conditions and extension of the disease [43]. Interestingly, also other non-neuroendocrine tumors are associated with altered circulating levels of CgA and/or its fragments, e.g. multiple myeloma and chronic lymphocytic leukemia [7, 8]. Thus, also non-neuroendocrine tumors can be exposed to circulating CgA and, consequently, to its vasoregulatory functions, despite the fact that they do not secrete it. Considering that tumors can produce proteases, circulating CgA may be fragmented when it reaches tumor tissues if sufficient amounts of proteases are produced. This hypothesis is supported by the results of a recent study in patients with multiple myeloma [8]. Interestingly, some patients with normal levels of circulating CgA (measured with classical assays unable to discriminate between full-length CgA and fragments) actually showed reduced levels of full-length CgA and higher levels of CgA fragments, when specific assays were used. In particular, a conspicuous degree of CgA fragmentation in the C-terminal region was observed in these patients, compared to healthy subjects [8]. Notably, the balance of pro-/anti-angiogenic fragments (discussed above, e.g. $CgA_{1–439}$, $CgA_{1–373}$, $CgA_{1–76}$, and other fragments) was tipped toward a pro-angiogenic state in their bone marrow plasma, suggesting that a proteolytic mechanism capable of "turning-on" the CgA-angiogenic switch was active in these patients [8]. Accordingly, the ratio between pro- and anti-angiogenic forms correlated with the bone marrow plasma levels of VEGF and FGF2, two important pro-angiogenic factors, and with the tumor microvessel density [8]. These findings suggest that fragmentation of circulating CgA can indeed occur in the tumor microenvironment, likely because of local proteases. Whether detection of CgA levels and its fragmentation has a prognostic value in multiple myeloma and other non-neuroendocrine tumors remains an open question. Another central question is whether CgA molecules brought to tumors by the bloodstream might affect tumor growth. Possibly, while physiological levels of full-length CgA are likely crucial for maintaining the vascular homeostasis in physiological conditions, the unbalanced production of pro-/anti-angiogenic CgA polypeptides, as observed in multiple myeloma patients, might contribute to sustain local angiogenesis and tumor growth. Also, this hypothesis merits further investigation.

Chromogranin A in cardiovascular and inflammatory diseases

The levels of circulating CgA and its degree of fragmentation can be altered also in certain cardiovascular and inflammatory diseases, which will be discussed in the following paragraphs.

Heart failure

Chronic heart failure is characterized by impaired cardiac contractility, activation of the neuroendocrine system, and release of inflammatory cytokines. In 2000, we have shown that circulating CgA is increased in patients with chronic heart failure depending on the severity of the disease and that it was an independent predictive factor for mortality [16, 22]. Interestingly, while CgA did not correlate with hormones typically activated in chronic heart failure, such as catecholamines, vasopressin, endothelins, and components of the renin-angiotensin-aldosterone system, it correlated with soluble TNF receptors, a sensitive marker of systemic inflammation. Later studies supported the concept that elevated plasma levels of CgA in chronic heart failure are related to activation of an inflammatory response more than to activation of the sympatho-adrenergic system [55]. Tissue expression studies showed that the heart itself could contribute to the increased circulating pool of CgA [80]. Indeed, studies on the expression of CgA in cardiac ventricle biopsies from patients with dilated and hypertrophic cardiomyopathy showed that CgA is co-localized with brain natriuretic peptide (BNP) in

cardiomyocyte secretory granules [80]. Furthermore, blood levels of CgA were strongly associated with those of BNP, an important diagnostic and prognostic marker of heart failure, and with left ventricular end diastolic pressures [80]. These results suggest that (a) these hormones are co-released in the circulation and (b) cardiomyocyte stretching might represent an important mechanism for the up-regulated expression of both hormones [80]. A later study in patients with acute destabilized heart failure showed that CgA adds independent prognostic information to amino-terminal proBNP, another established prognostic marker of the disease, indicating an improved prognostic ability of the combination of the two markers [29]. However, overall, the prognostic value of CgA in acute and chronic myocardial failure is still a matter of debate [39]. Indeed, results from the GISSI (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico) trial, conducted on 1233 patients with chronic stable heart failure, showed that CgA was associated with all-cause mortality or cardiovascular morbidity in univariate but not in multivariate regression analysis adjusted for known risk factors [83].

Besides CgA, also the catestatin fragment has been reported being increased in the bloodstream of patients with chronic heart failure, especially those with ischemic etiology, in relationship with the severity of the disease [57]. Catestatin values remained elevated in these patients when symptoms were alleviated. A recent study conducted on 202 patients with chronic heart failure showed that catestatin was an independent and strong risk factor for all-cause mortality and cardiac death [78]. In another study in patients with acute heart failure, catestatin did not provide independent prognostic information, whereas CgA levels did [74]. A novel aspect that emerged from this study is a reduced CgA-to-catestatin conversion in patients with chronic heart failure. Patients with low CgA-to-catestatin conversion had a worse outcome in terms of survival. The authors provided experimental data to support the hypothesis that reduced processing of CgA was due to the presence of a hyper-glycosylated form of CgA in the failing heart. Reduced CgA-to-catestatin conversion could be detrimental since catestatin can reduce diastolic calcium leak from the sarcoplasmic reticulum by inhibiting calcium/calmodulin-dependent protein kinase II-delta activity [74]. Overall, catestatin levels appear to be different in acute and chronic heart failure, and this may impact on the prognostic significance.

Differently from CgA and catestatin, vasostatin-II serum levels were reduced in patients with ischemic chronic heart failure [75]. In these patients, vasostatin-II was negatively and independently associated with major adverse cardiac effects, defined as re-infarction, revascularization, cardiovascular death, and hospitalization for heart failure. Interestingly, this study also showed that vasostatin-II can improve cardiac function and reduce remodeling, fibrosis, and inflammation in a rat model of myocardial infarction, suggesting that the reduced levels of this fragment may have a pathogenic role in heart failure.

Acute myocardial infarction

Established markers for diagnosis and long-term prognosis of acute myocardial infarction are troponins, released during cell lysis, and brain natriuretic peptides released from cardiac granules. The first observation on a positive association of CgA with acute myocardial infarction was reported by Omland et al. in 2003 [73]. Elevated plasma CgA, 3 days after the onset of symptoms, was associated with increased risk of death in patients with documented acute myocardial infarction. In a later study performed in 217 patients with complicated myocardial infarction, CgA was a strong, independent prognostic indicator of risk of death or hospitalization for heart failure [32]. The largest study on CgA in acute coronary syndromes included 1268 patients [52]. In this study, serum CgA concentrations measured 1 day after patient's admission were strongly associated with increased long-term mortality and heart failure hospitalizations, but not with recurrent myocardial infarction [52]. Another study showed that CgA can contribute to a cluster of nine biomarkers that represent a strong independent predictor of all-cause death in acute myocardial infarction [64].

Catestatin is increased after acute myocardial infarction [102]. Interestingly, the blood levels of catestatin, a peptide capable of reducing catecholamine release in the blood by inhibiting nicotinic acetylcholine receptor, negatively correlated with the levels of circulating noradrenaline. A pathophysiological role of catestatin in this disease is suggested by the observation that administration of exogenous catestatin to rats subjected to myocardial infarction exerts cardioprotective effects by decreasing the cardiac sympathetic drive and impairing autonomic function [79, 101]. Increased circulating catestatin levels were also correlated with the occurrence of malignant arrhythmia after acute myocardial infarction [77]. At variance with these results, a recent report showed reduced plasma catestatin levels in patients with acute coronary syndrome and unstable angina, compared to patients without a diagnosis of coronary artery disease and no association with major adverse cardiac events [104].

Hypertension

CgA is essential for the correct storage of catecholamines in secretory granules, while catestatin, by virtue of its inhibitory activity on nicotinic cholinergic receptors, represents a negative feedback control mechanism against the excessive release of catecholamines in the blood [63]. Accordingly, CgA knockout mice display increased plasma catecholamine levels and hypertension, which is reverted by infusion of catestatin [61]. Interestingly, CgA levels are increased in essential and secondary

hypertension [72], whereas plasma catestatin levels are reduced in hypertensive patients and even in their normotensive offspring, compared to controls (as measured by radioimmunoassay) [71]. However, another study showed that catestatin, as measured by ELISA, is increased in patients with essential hypertension [66]. Recently, the catestatin Gly364Ser allele was associated with enhanced risk for hypertension in Indian and Japanese populations [18, 54]. On the other hand, a previous study had shown that subjects with this variant have lower diastolic blood pressure than subjects with the wild-type gene [82]. Moreover, a humanized CgA mouse model bearing a catestatin polymorphism corresponding to Gly364Ser demonstrated a reduced response to stress and lower catecholamine levels, suggesting a reduced risk to develop hypertension [67]. These discrepant reports might reflect the use of different assays and point to the need for further studies in larger patient populations.

Atherosclerosis and other inflammatory diseases

We have recently shown that blood levels of total-CgA and $CgA_{1–76}$ (vasostatin-I) correlate with the progression of carotid artery atherosclerosis in asymptomatic patients [3]. In these patients, vasostatin-I was an independent predictor for maximum plaque size, while full-length CgA was associated with lipid-rich and hypoechoic plaques. Other investigators have shown that reduced levels of $CgA_{1–113}$ (vasostatin-II) are present in serum and peripheral blood mononuclear cells of patients with severe coronary artery atherosclerosis, compared to patients with normal coronary arteries or less severe disease, suggesting the potential utility of this fragment as a marker of atherosclerotic risk [59]. Interestingly, vasostatin-II can attenuate atherosclerosis in $ApoE^{(-/-)}$ mice fed with a high-fat diet, an established animal model for atherosclerosis, and reduce infiltration of monocytes/macrophages [103], suggesting a pathophysiological function of vasostatin-II in this disease.

Several studies demonstrated the diagnostic and prognostic values of CgA in critically ill patients and in severe sepsis [48, 84, 107]. One study showed that plasma vasostatin-I was more sensitive and specific than plasma CgA to diagnose sepsis and to assess its severity [19]. The presence of a CgA gene polymorphism, namely the CgA-415 T/C mutant genotype, was associated with a higher mortality in critically ill patients [56].

Elevated CgA levels were observed also in patients with other inflammatory diseases, such as giant cell arteritis, Takayasu's arteritis, Erdheim-Chester disease, inflammatory bowel disease, and rheumatoid arthritis [15, 27, 28, 34, 89, 97]. A direct relationship between blood levels of CgA and TNF has been demonstrated in patients with rheumatoid arthritis or Erdheim-Chester disease, suggesting a link between inflammation and CgA secretion [28, 34]. Although high serum levels of CgA were significantly associated with severe extra-articular manifestations in rheumatoid arthritis, CgA as well as vasostatin-I could protect endothelial cells in vitro from TNF-mediated activation [28] and vascular leakage [35] suggesting a protective rather than a pathogenic role for CgA and vasostatin-I in inflammatory conditions. It is therefore possible that CgA and vasostatin-I represent counter-regulatory mechanisms. In Takayasu's arteritis, reduced blood levels of the anti-angiogenic CgA peptides (such as vasostatin-I) were associated with arterial remodeling [97], whereas in giant cell arteritis, high levels of CgA reflected persistent arterial inflammation [27].

Other pathologies

The presence of CgA in the secretory granules of endocrine cells in the pancreas and the metabolic effects of its fragments suggest that CgA may have a pathophysiological role in diabetes, and that it could be a potential diabetes biomarker [14]. However, no clear consensus is present in the literature regarding the role of CgA as a diabetes biomarker. In any case, higher pancreastatin levels were found in gestational diabetes, and a role in insulin resistance has been proposed for this peptide [14].

A marked accumulation of plasma CgA can also occur in patients with renal failure [47]. Furthermore, high plasma catestatin levels are associated with increased risk for cardiac death in hemodialysis patients [92]. Variations of CgA plasma levels, but not of vasostatin-I, were observed also in patients with active Meniere's disease, depending on the frequency of vertigo spells and the time from the last crisis [95].

Conclusions and perspectives

Based on the reviewed literature, CgA and some of its fragments appear to be established or potential biomarkers for a variety of neoplastic, cardiovascular, and inflammatory diseases. However, because of the high heterogeneity of the CgA antigen, owing to differential post-translational modification and proteolytic processing, a series of factors should be considered when comparing the results of different studies or planning future investigations. A first issue to consider is the biological matrix where CgA is analyzed. Plasma appears to be better than serum since thrombin, a proteolytic enzyme activated during the clotting process, can cleave CgA into fragments [23]. This may lead to an artificial increase of certain fragments and to the loss of other important ones. A second issue to consider is the impact of therapy on the circulating pool of CgA and its fragments in healthy and pathological conditions, given that certain drugs, e.g. proton pump inhibitors (PPIs), can enhance the circulating levels of CgA (discussed above). Thus, it would be important to know which fragments are released in circulation by PPIs in normal and pathological conditions and to assess whether PPI-independent, disease-specific fragments exist. A third issue regards the type of immunoassay that should be used to detect specific fragments or the total pool of circulating CgA forms (total-CgA). For the

latter purpose, immunoassays based on antibodies that recognize the central part of human CgA appear a good choice because this region is less susceptible to proteolysis. Consequently, intact molecules and major fragments are likely detected. However, these assays can still miss some important fragments and, like most CgA immunoassays, might be difficult to standardize because immune-reactive species in standard solutions and samples might be different. An interesting alternative is to use a processing-independent assay (PIA), an assay based on production of a uniform peptide fragment (by sample treatment with trypsin) that is easier to detect and standardize [13, 40]. Remarkably, this assay can detect also epitopes hidden in the precursor and can provide information on CgA products irrespective of original processing. However, to fully assess the diagnostic/prognostic utility of the CgA system, these assays should be complemented with other assays selective for the precursor or its fragments. For example, sandwich ELISAs for full-length $CgA_{1–439}$, $CgA_{1–373}$, $CgA_{1–78}$, or $CgA_{1–76}$ have been developed using a cross-reactive antibody against the N-terminal region in the capture step and different fragment-specific antibodies (against the C-terminal residues of each fragment) in the detection steps [8, 23]. Other assays with region-specific antibodies have been also developed [91]. As some fragments of CgA can exert opposite biological effects (e.g. anti- and pro-angiogenic), the relative levels and not only the absolute values should be taken into consideration for assessing their diagnostic/prognostic utility [8]. Finally, considering the CgA gene polymorphisms and the various post-translational modifications of CgA (e.g. glycosylation and phosphorylation), which may impact on CgA activity and processing, development of new assays is required for monitoring these modifications in healthy and pathological conditions. In summary, CgA and its fragments represent a very complex system both in terms of structures and biological functions. Although the relevance of CgA as a disease biomarker has been widely demonstrated, particularly in neuroendocrine tumors, it appears that more efforts aimed at investigating the key roles of the proteolytic processing and post-translational modifications on CgA bioactivity and at detecting its various fragments in tissues and biological fluids are necessary for thoroughly assessing its pathophysiological function and its value as diagnostic/prognostic marker.

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