Chromogranin A, a Member of Neuroendocrine Secretory Proteins as a Selective Marker for Laboratory Diagnosis of Pheochromocytoma.

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Summary

The function of chromogranin A (CGA) is reviewed, and the radioimmunometric determination of plasma CGA was evaluated as a marker of pheochromocytoma using comparison of pheochromocytoma patients immediately before surgery (group P, n=25, 635±451 ng/ml) with other groups of patients, i.e. pheochromocytoma patients approximately 1 year after removal of tumor (group PP, n=13, 69±33 ng/ml), medullary thyroid carcinoma patients (group M, n= 22, 106±59 ng/ml), congenital adrenal hyperplasy patients (n=33, 65±40 ng/ml), and controls (n=31, 66±29 ng/ml). A CGA above cut off value 130 ng/ml was found in 24 of 25 patients in group P, 1 (relapse) of 13 patients in group PP, and 4 of 22 patients in group M. In the group P we found a significant association between the size of the tumors removed and plasma CGA concentrations (p=0.0016), and also a statistically significant (p=0.0016) relationship between plasma CGA concentrations and PASS score rating the malignity of pheochromocytoma. We can conclude that plasma CGA concentration as determined by radioimmunometric assay (which is simple without the necessity of special laboratory equipment) is an effective marker of pheochromocytoma with association to malignity and tumor mass.

Keywords
chromogranin A - pheochromocytoma - radioimmunometric assay - plasma

Introduction

Pheochromocytoma, which is a rare catecholamine-producing tumor with preferential localization in the adrenal gland, arises from neuroendocrine chromaffin cells of the adrenal medulla (Guller *et al.* 2006). It is included in the heterogenous group of neuroendocrine tumors together with carcinoid, gastroenteropancreatic neuroendocrine tumors, pituitary tumors and medullary carcinoma of the thyroid. These tumors originate in neuroendocrine cells characteristic with their production of neurotransmitters, neuromodulators and neuropeptide hormones, with a presence of dense-core secretory granules, and an absence of axons and synapses (Barakat *et al.* 2004). However, extraadrenal pheochromocytomas have been also described in locations such as the Zuckerkandle organ, bladder, sacrum, testis, rectum, pelvic floor, the upper abdomen in association with celiac, superior mesenteric, and inferior mesenteric ganglia, the thorax, and the neck, most commonly in association with the ninth or tenth cranial nerve ganglion (Frezza *et al.* 2002). The incidence of pheochromocytoma is similar in both sexes and most frequent between the ages of thirty and fifty. Multiple and bilateral pheochromocytomas constitute 5 to 10 percent of all cases. Pheochromocytoma occurs sporadically or is related to family syndromes such as: syndrome of multiple endocrine neoplasia (MEN), neurofibromatosis, von Hippel-Lindau's disease, Sturge-Weber's syndrome, and tuberous sclerosis. Cases in families with genetic predisposition usually occur at a young age and are mostly bilateral and with more aggressive biological behaviour (Tatic *et al.* 2002).

Chromaffin cells of the adrenal medulla contain chromogranin A (CGA) which is co-stored and co-released with catecholamines (epinephrine, norepinephrine) from storage granules in the adrenal medulla, plays a catalytic role in granule biogenesis (Mosley *et al.* 2007). Murray *et al.* (1987) traced human CGA to chromosome 14 by probing DNA from a hybrid cell panel with specific cDNA. Konecki *et al.* (1987) isolated a full-length clone

encoding human CGA from a lambda-gt10 cDNA library of a human pheochromocytoma. The nucleotide sequence showed that human CGA is a 439 amino acid protein preceded by an 18-residue signal peptide. It was found (Wu *et al.* 1991) that the CGA gene has 8 exons and 7 introns spanning about 11 kb.

CGA is a member of granins (chromogranins or secretogranins), which are a family of acid proteins present in the secretory granules of a wide variety of endocrine and neuro-endocrine cells with numerous pairs of basic amino acids as potential cleavage sites (Konecki *et al.* 1987). Granins seem to be the precursors of biologically active peptides, which may act as helper proteins in the packaging of peptide hormones and neuropeptides. Granins exert similar subcellular location. Their structural homology is limited to the short conserved C-terminal region. The granin family of acid secretory proteins consists of the 3 „classic“ granins - chromogranin A, which was first isolated from chromaffin cells of the adrenal medulla, chromogranin B (secretogranin I, 657 amino acids long protein) initially characterized in a rat pheochromocytoma cell line, and chromogranin C (617 amino acids) also known as secretogranin II, which was originally described in the anterior pituitary (Taupenot *et al.* 2003). 4 other acidic secretory proteins considered to be the members of the granin family are secretogranin III (Ottiger *et al.* 1990), secretogranin IV (Krisch *et al.* 1986), secretogranin V (Mbikay *et al.* 2001), and secretogranin VI (Ischia *et al.* 1997).

Biological function of CGA was investigated using CGA-null mice (Mahapatra *et al.* 2005). The following were found in these animals: a decrease in chromaffin granule size and number, increase in blood pressure, loss of diurnal blood pressure variation, increase in left ventricular mass and cavity dimensions, decrease in adrenal catecholamine and neuropeptide Y contents, and an increase in plasma catecholamine and neuropeptide Y concentrations. The authors concluded that CGA has a definitive role in the autonomic control of circulation.

CGA was found to be an “on/off” switch, which is sufficient by itself to drive dense-core secretory granule biogenesis and hormone sequestration in endocrine cells (Kim *et al.* 2001).

CGA is a precursor of biologically active peptides, which act as autocrine or paracrine negative modulators of the neuroendocrine system. To these peptides belong vasostatin 1 with antiadrenergic effects (Gallo *et al.* 2007), pancreastatin, which is a strong inhibitor of glucose induced insulin release from the pancreas (Helman *et al.* 1988), parastatin (parathyroid secretory protein) inhibiting low Ca^{2+}-stimulated parathyroid secretion (Fasciotto *et al.* 1993), and catestatin, which is a catecholamine release-inhibitory peptide (Rao *et al.* 2007, Taylor *et al.* 2000). Knowledge is limited about other peptides derived from CGA such as chromostatin, beta-granin (vasostatin 2), WE-14, GE-25 etc. The proteolysis of CGA is tissue specific, for example pancreastatin was found in alpha cells of pancreas, chromostatin in beta cells of pancreas, but neither pancreastatin nor chromostatin were observed in adrenal chromaffin cells (Cetin *et al.* 1993).

The primary structure of the CGA molecule prepared by authors according to the ExPASy proteomics server (Gasteiger *et al.* 2003) of the Swiss Institute of Bioinformatics (access number P10645) is shown in Fig. 1 with mapped CGA-derived peptides and potential glycosylation and phosphorylation sites. 3-Dimensional structural data on CGA is lacking and only catestatin was determined experimentally using nuclear magnetic resonance by Preece *et al.* (2004). The model of catestatin is also shown in Fig. 1, the 3D-structure of which was constructed by authors using CAChe software (Fujitsu Ltd, Japany) according the XYZ coordinates from Protein Data Bank file (www.rcsb.org/pdb, code 1LV4.pdb).

CGA is secreted by a great variety of peptide-producing endocrine neoplasms such as pheochromocytoma, parathyroid adenoma, medullary thyroid carcinoma, carcinoids, oat-cell lung cancer, pancreatic islet-cell tumors, and aortic-body tumor (O'Connor and Deftos 1986). Nobels *et al.* (1997) evaluated the clinical usefulness of CGA as a neuroendocrine serum

marker. CGA was increased in 50 % of patients with neuroendocrine tumors (n=211) and most frequently increased in subjects with gastrinomas (100%), pheochromocytomas (89%), carcinoid tumors (80%), nonfunctioning tumors of the endocrine pancreas (69%), and medullary thyroid carcinomas (50%). The highest levels of CGA were observed in subjects with carcinoid tumors. Elevated concentrations of CGA were present in 7 % of control subjects. Nobels *et al.* (1997) concluded that CGA was the best general neuroendocrine serum marker available at that time. In a group of 63 patients with histologically-proven pheochromocytoma (52 pheochromocytomas and 14 paragangliomas, 14 of the patients had a family history of the disease) a CGA assay was highly efficient in diagnosing pheochromocytomas in the absence of renal insufficiency (d'Herbomez *et al.* 2007). The evaluation of the CGA immunoradiometric assay (IRMA) in the selection of patients affected by adrenal incidentaloma (n=144) for accurate but high-cost and time-consuming ^{123}I-metaiodobenzyl-guanidine (MIBG) scintigraphy was completed by Giovanella (2005). Circulating CGA concentration was positive in 12 out of 12 patients with pheochromocytoma and negative in 92 out of 92 patients with non-chromaffin adrenal nodules. Feng *et al.* (2005) described that CGA was detected in all pheochromocytomas (n=25), and exhibited less or no expression in adrenocortical tumors.

A significant positive relationship was demonstrated between tumor mass and serum CGA levels (d'Herbomez *et al.* 2001, Giovanella *et al.* 2006, Nobels *et al.* 1997). An additional important finding is the existence of the statistically significant difference of CGA expression between benign and malignant pheochromocytomas (Feng *et al.* 2005, Portela-Gomes *et al.* 2004). Plasma CGA was progressively higher (probability level $p<0.0001$) from control subjects (48.0±3.0 ng/ml) to benign pheochromocytoma (n=13, 188±40.5 ng/ml) to malignant pheochromocytoma (n=14, 2932±960 ng/ml), i.e. markedly elevated chromogranin A may point to malignant pheochromocytoma (Rao *et al.* 2000).

When comparing CGA with other tests used for laboratory diagnosis of pheochromocytoma (urinary epinephrine, norepinephrine, vanillylmandelic acid and metanephrines determined by high performance liquid chromatography) the immunoradiometric measurement of serum CGA concentrations showed a higher sensitivity, specificity and accuracy, and can be used as a single test for the diagnosis of pheochromocytoma (Giovanella and Ceriani 2002). In pheochromocytoma patients (n=11) immunoradiometric assays based on monoclonal antibodies (CgA-RIA CT, CIS bio international) before surgery and again four weeks after tumor removal showed that plasma CGA concentrations determined significantly decreased ($p<0.001$) (Cotesta *et al.* 2005). Measuring plasma CGA is a useful adjunctive test when evaluating patients with adrenal incidentalomas and presumed pheochromocytomas CGA (Giovanella and Ceriani 2002). Patients with adrenocortical tumors usually do not have elevated levels of plasma CGA (Bernini *et al.* 2004).

In this study we presented our experiences with immunometric determination of plasma CGA in groups of patients who suffered of pheochromocytoma (PHEO), multiple endocrine neoplasia (MEN), medullary thyroid carcinoma (MTC), and congenital adrenal hyperplasia (CAH). Patients with various endocrine disorders other than PHEO, MEN, MTC, and CAH were used as controls.

Methods

Groups of patients

Plasma CGA was determined in pheochromocytoma patients aged 47 ± 14 (mean ± standard deviation) years (group P, n=25, 13 males, 12 females) of them were 3 MEN II patients with medullary thyroid carcinoma (1 male, 2 females aged 32 ± 11 years) immediately before surgery, and approximately 1 year after removal of tumor (group PP,

n=13, 7 males, 6 females). Group M contained MTC (medullary thyroid carcinoma without pheochromocytoma) patients aged 45 ± 21 years (n=22, 9 males, 13 females), group H were CAH (congenital adrenal hyperplasy) patients aged 22 ± 9 years (n=33, 17 males, 16 females). Controls (group C, n=31, 8 males, 23 females, aged 47 ± 18 years) consisted of patients without pheochromocytoma, but who had underwent surgery of adrenal gland because of non-functioning adrenal masses (n=11, 3 males (age 49 ± 12 years), 8 females (age 43 ± 17 years)). The control group additionally was made up of 7 patients with various adrenal diseases (no pheochromocytoma), 4 hypertensive patients, and 9 patients with thyroid disorder of various etiology (no MTC). Malignity of the surgically removed pheochromocytoma tumors was determined according the PASS score, which has been suggested for biological assessment of pheochromocytomas (Thompson 2002).

Immunoassay of CGA

A solid-phase two-site immunoradiometric assay was used with primary immobilized monoclonal antibodies and secondary radioiodinated monoclonal antibodies, both directed against the central domain of the molecule (145-245), which is less sensitive to proteolysis (manufacturer CIS bio international, France; code CGA-RIACT). CGA was measured in 50 µl EDTA - plasma samples. The reference range according to the manufacturer was 20-150 ng/ml for plasma EDTA samples, and 19.4 - 98.1 ng/ml for serum samples. The capacity of the kit is 42 samples in duplicate, analysis takes two days. Characteristics of the immunometric assay are: intraassay precision 3.8 % CV (coefficient of variation), interassay precision 5.7 % CV, detection limit 1.5 ng/ml. The concentration of CGA can be increased due to renal failure, hypergastrinemia, or steroid treatment (data of CIS bio international).

Results

Plasma CGA concentrations are shown in Fig. 2 as a black points, the data for MEN patients is highlighted with a red color. The decrease of CGA concentrations (mean ± standard deviation (sd)) between groups P (pheochromocytoma) and PP (post-pheochromocytoma) is also shown in the Fig. 2. The mean concentrations of plasma CGA ± sd were 635 ± 451 ng/ml in group P (pheochromocytoma), 69 ± 33 ng/ml in group PP (post-pheochromocytoma), 106 ± 59 ng/ml in group M (medullary thyroid carcinoma), 65 ± 40 ng/ml in group H (congenital adrenal hyperplasy), and 66 ± 29 ng/ml in controls (group C). These statistically significant differences between group P (pheochromocytoma) and all other groups ($p<0.0001$) were found using the Student T-test. Group M (medullary thyroid carcinoma) also showed statistically significant differences in comparison with all other groups ($p<0.05$). Statistically significant differences were not found among groups PP (post-pheochromocytoma), group H (congenital adrenal hyperplasy), and group C (controls). We did not find an association of CGA concentration with age in pheochromocytoma patients or any other group, whether patients or controls. A statistically significant association concerning the size of the removed tumor and CGA concentration was found in group P (pheochromocytoma), where plasma CGA is dependent on the mass of the removed tumor ($p=0.0016$) according the equation: CGA (ng/ml)=252.ln[weight (g)]–524. Pearson correlation coefficient for this equation has a value of 0.6319, Spearman correlation coefficient is 0.7942, and R-squared is 0.3993. The association between CGA and mass of the removed tumor is shown in Fig. 3. We found also an association of CGA concentration with PASS score (malignity), which was statistically significant ($p=0.0016$). The equation of this dependence is: CGA(ng/ml)=126.PASS+29, where the Pearson correlation coefficient is 0.6324, Spearman correlation coefficient is 0.7158, and R-squared is 0.4000. The association between CGA and PASS is shown in Fig. 4.

Discussion

CGA immunoradiometric assay employs a simple and feasible technology. It was found (Giovanella *et al.* 2006) that this kind of CGA determination in plasma or serum is as sensitive as, and slightly more accurate than HPLC determined plasma metanephrines in chromaffin-tumors detection. Currently, two different methods for assaying CGA, immunoradiometric assay (IRMA) and enzyme-linked immunosorbent assay (ELISA) are widely used in routine practice. When comparing of these assays, the coefficients of variation increased approximately five-fold when shifting from the IRMA to the ELISA method (Verderio *et al.* 2007). In addition, no antihypertensive drugs interfered with the analysis of CGA levels. However, some false positive results have to be mentioned in the presence of renal impairment, hypergastrinemia, and corticotherapy (Biausque et al. 2003, Hsiao et al. 1991).

Our results concerning the measurement of circulating CGA are in agreement with the fact that plasma CGA is elevated in pheochromocytoma patients. Statistically significant differences in circulating CGA among these patients and other groups in the study uniquely determine the plasma CGA as a suitable marker of pheochromocytoma. Post-operative levels of plasma CGA determined at least one year after tumor removal (group PP) were normal with one exception - a relapse of pheochromocytoma was diagnosed in this one patient. From this point of view, the repeated measurement of circulating CGA over a period of months following removal of a tumor is a good indicator of remise or relapse of the disease.

Elevated plasma CGA concentrations were observed in part of the MTC patients, and group M has also shown statistically significant differences in comparison with all other groups. This is in accordance with the fact that the increased concentrations of circulating CGA were found not only in pheochromocytoma but also in other species of neuroendocrine

tumors. Medullary thyroid cancer (MTC) shares biochemical features with these tumors but the particular characteristics are largely unexplored. For example serum CGA was increased in 12 of 45 medullary thyroid carcinoma patients with elevated calcitonin levels and in 4 of 16 medullary thyroid carcinoma patients with undetectable calcitonin levels (Guignat *et al.* 2001). It is important to note the finding (de Groot *et al.* 2006) that in addition to plasma calcitonin, only carcinoembryonic antigen and CGA could differentiate between stable and progressive MTC, and both are most useful markers in the follow-up of MTC. From our results (Fig. 2) it is evident that increased concentrations of plasma CGA were found in 18 % (4 from 22) of MTC patients. From this point of view CGA is not a suitable marker of MTC, but it is important for detection in MEN patients where MTC is together with pheochromocytoma as part of the syndrome. The increased concentrations of plasma CGA in the rest of patients (groups H, C) were negligible (1 patient in group H under a corticosteroid treatment).

There are other situations where circulating CGA is increased. The high levels of CGA correspond with serious diseases found in human subjects. Immunoreactive CGA was observed not only in adrenal chromaffin cells but also in alpha cells of the pancreas (Cetin *et al.* 1993). Positive and negative predictive values of circulating CGA were 84 and 78% respectively (90% specificity and 68% sensitivity) for pancreatic neuroendocrine tumors (Zatelli *et al.* 2007). Neuroendocrine differentiation in prostate cancer, promoted by neuroendocrine cell secreted products including CGA, appears to be associated with tumor progression, poor prognosis, and hormone-refractory disease (Adolf *et al.* 2007). Plasma CGA levels in prostate cancer increase with the severity of the disease and were associated with a poor survival prognosis in patients (Hirano et al. 2007). CGA has also been associated with some non-neuroendocrine tumors and it was elevated in 34/72 patients with breast cancer, 11/21 with lung cancer, 10/28 with gastrointestinal cancer, 7/12 with gynaecological

cancer, 6/9 with genitourinary cancer, 5/5 with haematological cancer, and 3/4 with head and neck cancer (Tropea *et al.* 2006). CGA is produced by human myocardium and exerts negative inotropic and lusitropic effects on mammalian heart. Patients with dilated cardiomyopathy or hypertrophic cardiomyopathy showed increased CGA plasma levels, an increase, which was not found in controls (Pieroni *et al.* 2007).

Increased levels of CGA correlate with pheochromocytoma tumor mass. The correlation is lower in large tumors with mass available over 400 g (Fig. 3), we therefore used the logaritmic values of both CGA and mass of the removed tumors. The explanation for this lower correlation may be found in pathology, since extremely large tumors are frequently filled, at least partially, with old blood clots or old posthemorrhagic cysts and the amount of functioning tissue is proportionally smaller. The association of CGA concentration with PASS score (malignity) in pheochromocytoma patients (Fig. 4) is very important, since it supports the predictivity of chromogranin A as a malignant marker.

We can conclude that plasma CGA concentration is an effective marker of pheochromocytoma with association to the malignity and tumor mass. The radioimmunometric determination of CGA is simple without the necessity of special laboratory equipment. Generally, the increased level of circulating CGA can also be an indicator of other serious diseases of the neuroendocrine system, including non-neuroendocrine tumors and cardiomyopathy. We further expect that simultaneously determined plasma CGA and free metanephrines can substantially enrich laboratory diagnosis of pheochromocytoma.

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Figure 1. The primary structure of the chromogranin A (CGA) according the ExPASy proteomics server (Gasteiger, *et al.*, 2003) of the Swiss Institute of Bioinformatics (access number P10645) with mapping of biologically active peptides marked as a bold lined arrows. Potential glycosylation sites of CGA are marked yellow, potential phosphorylation sites are marked violet. Experimentally determined three-dimensional structure of catestatin, part of human CGA (amino acids 370 to 390) using nuclear magnetic resonance (Preece *et al.* 2004) is shown in the lower part of figure. Model of molecule was constructed using CAChe software (Fujitsu Ltd, Japany) according the XYZ coordinates from the Protein Data Bank file (code 1LV4.pdb).

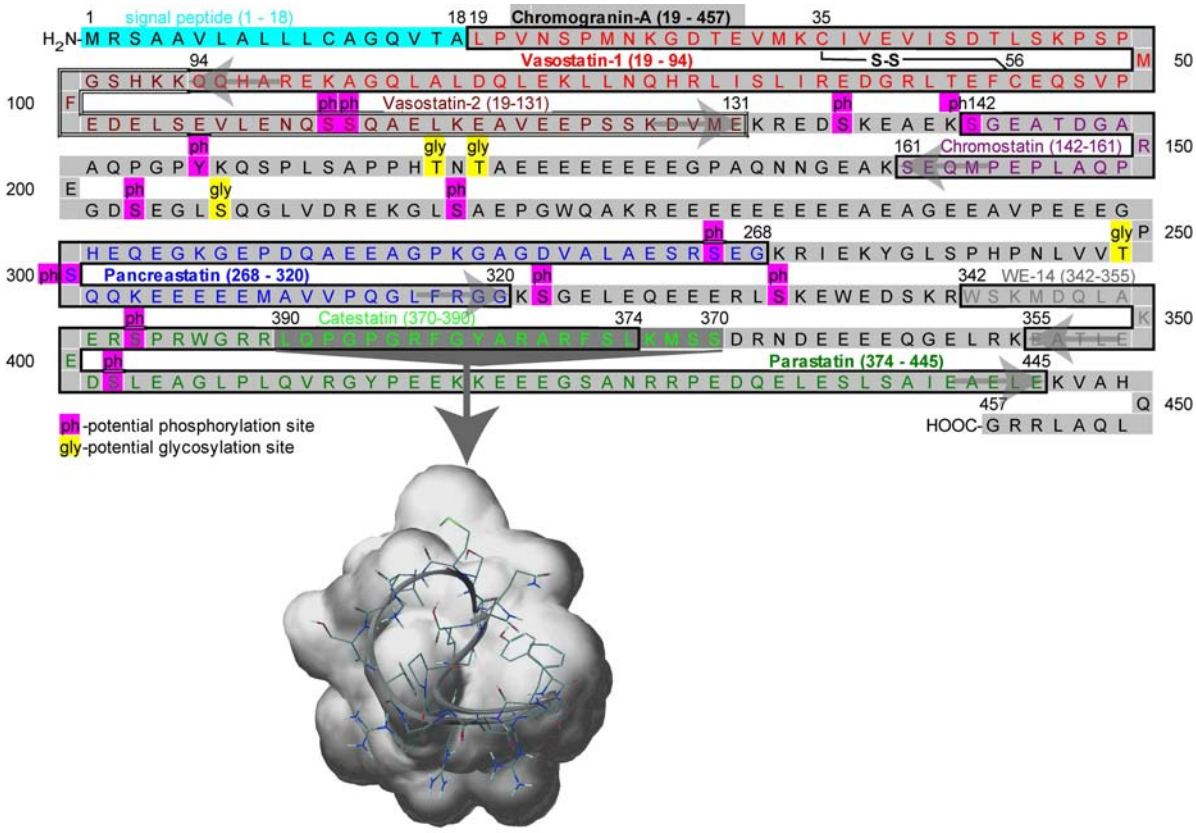

Figure 2. Plasma chromogranin A (CGA) in various groups of patients: P - patients with pheochromocytoma immediately before surgery (red points are MEN patients), PP - patients at least 1 year after surgery of pheochromocytoma (relapse of disease in 1 subject), M - medullary thyroid carcinoma patients, H - patients with congenital adrenal hyperplasy, C – controls (patients with various endocrine disorders, but no pheochromocytoma or MTC).

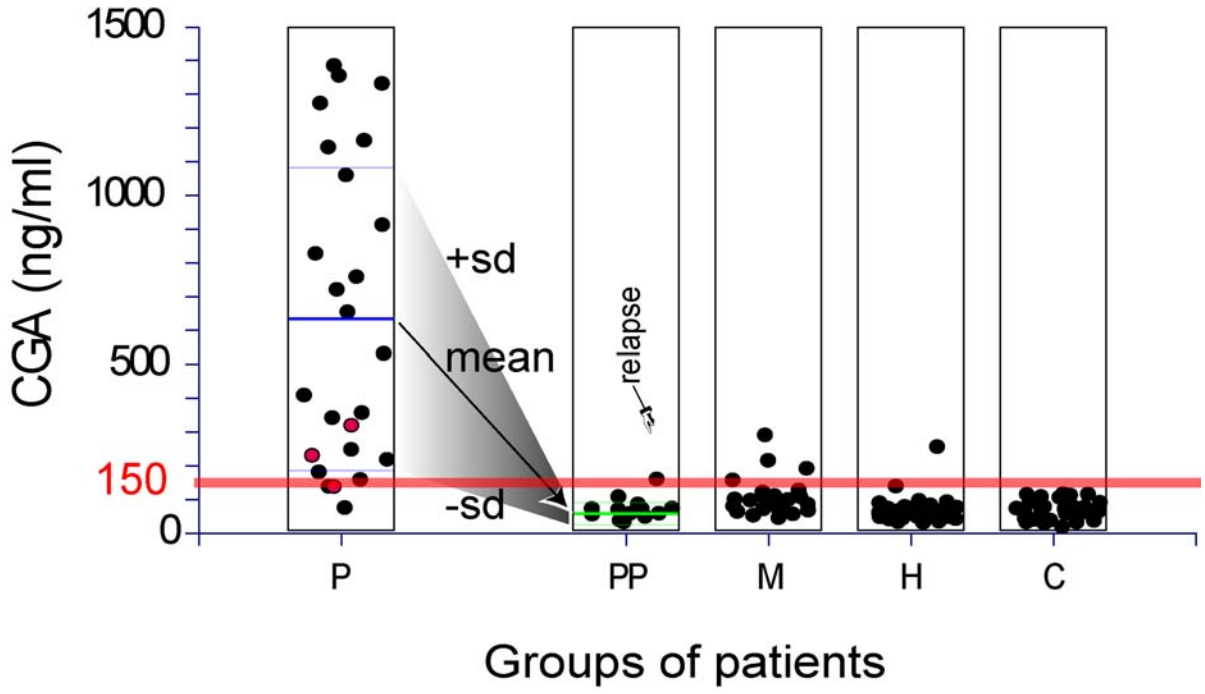

Figure 3. The association of plasma CGA concentration (group P - pheochromocytoma patients) with mass of removal tumour, which is described by the equation: CGA (ng/ml) = 252.ln[weight(g)]–524. In the figure are shown confidence limits and probability ellipse.

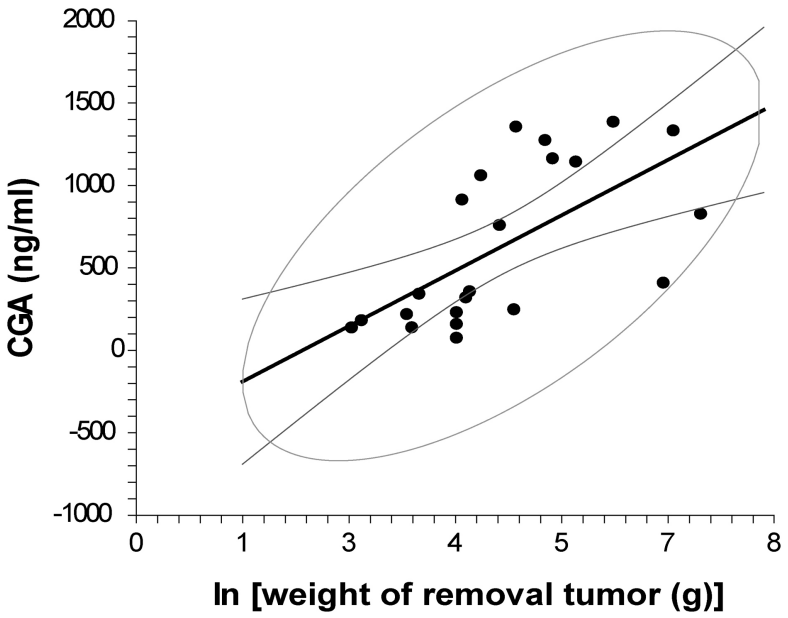

Figure 4. The association of plasma CGA concentration (group P - pheochromocytoma patients) with PASS score, which is described by the equation: CGA (ng/ml) = 126.PASS+29. In the figure are shown confidence limits and probability ellipse.