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[1]*INSERM U413, Laboratory of Cellular and Molecular Neuroendocrinology, European Institute for Peptide Research (IFRMP 23), University of Rouen, Mont-Saint-Aignan, France*
[2]*Division of Clinical Pharmacology and Toxicology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland*
[3]*Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal*
[4]*Department of Hypertension, Hôpital Européen Georges Pompidou, Paris, France*
[5]*Department of Endocrinology, Hôpital de Brabois, University of Nancy, Nancy, France*
[6]*Department of Zoology, University of Hong-Kong, Hong-Kong, People's Republic of China*

We have previously demonstrated that measurement of tissue concentration of the novel secretogranin II-derived peptide EM66 may help to discriminate between benign and malignant pheochromocytomas. The aim of the present study was to characterize EM66 in plasma and urine of healthy volunteers and pheochromocytoma patients, in order to further evaluate the usefulness of this peptide as a circulating marker for the management of the tumors. HPLC analysis of plasma and urine samples demonstrated that the EM66-immunoreactive material coeluted with the recombinant peptide. In healthy volunteers, plasma and urinary EM66 levels were, respectively, 2.6 (1.9–3.7) ng/ml and 2.9 (1.9–4.6) ng/ml. In patients with pheochromocytoma, plasma EM66 levels were 10-fold higher than those of healthy volunteers (26.9 (7.3–44) ng/ml), and returned to normal values after removal of the tumor. In contrast, urinary EM66 levels were not significantly different from those of healthy volunteers (3.2 (2.2–3.9) ng/ml). Measurement of total or free plasma metanephrines and 24 hr urinary metanephrines in our series of patients revealed that these tests, taken separately, are less sensitive than the EM66 determination. Pheochromocytes in primary culture secreted high levels of EM66, suggesting that the chromaffin tumor was actually responsible for the increased plasma peptide concentrations in the patients. These data indicate that EM66 is secreted in the general circulation and that elevated plasma EM66 levels are correlated with the occurrence of pheochromocytoma. Thus, EM66 is a sensitive plasma marker that should be considered as a complementary tool in the management of pheochromocytoma.

Key words: chromogranins; plasma marker; EM66; pheochromocytoma; secretogranin II; neuroendocrine tumor

Chromogranins (Cgs) constitute a family of acidic glycoproteins encompassing chromogranin A (CgA), chromogranin B (CgB) and secretogranin II (SgII) that are widely distributed in neuroendocrine tissues where they are packaged in secretory granules.[1] The physiological role of these proteins is unclear but a number of potential biological functions have been postulated, including regulation of secretory granule formation and production of bioactive peptides through proteolytic processing.[2] It has been demonstrated that Cgs and Cgs-derived peptides are secreted together with hormones and neurotransmitters and can be detected in blood by radioimmuno assay (RIA) techniques.[3–5] This observation combined with their ubiquitous distribution in neuroendocrine, endocrine and nervous tissues makes Cgs useful markers of secretion of normal and tumoral neuroendocrine cells. In particular, measurement of CgA levels in plasma can be used to diagnose or monitor the progression of neuroendocrine tumors.[6] The highest accuracy has been observed in tumors characterized by an intense secretory activity, although the specificity and sensitivity remain high also in nonfunctioning tumors.[7] However, CgA levels may also be elevated in patients with hyperplasia[3] and may therefore not be reliable for distinguishing neuroendocrine hyperplasia from adenoma or carcinoma. In addition, CgA measurement showed a low sensitivity in certain neuroendocrine tumors such as insulinomas, pituitary adenomas and medullary thyroid carcinomas.[8,9] Thus, measurement of other Cgs or Cgs-derived peptides may be helpful for the diagnosis of different neuroendocrine tumors.

It has been reported that plasma levels of GAWK and CCB, 2 CgB-derived peptides, are elevated in patients with pancreatic islet-cell tumors[10,11] or with bronchial tumors.[12] Similarly, enhanced plasma concentrations of the SgII-derived peptide secretoneurin (SN) are associated with various endocrine tumors such as gastroenteropancreatic endocrine tumors or oat cell lung carcinomas, and are also related to the progression of prostatic carcinomas.[5,13] SgII is the precursor of 2 highly conserved peptides, *i.e.* SN and a 66-amino-acid C-terminal flanking peptide termed EM66.[14] Using specific polyclonal antibodies directed against recombinant EM66, we have previously shown the presence of EM66 in the rat pituitary and adrenal glands,[15] and in chromaffin cells of fetal and adult human adrenals.[16] We have recently demonstrated the occurrence of EM66 in human pheochromocytomas and found that low tissue concentrations of the peptide are associated with malignant differentiation of the tumor while benign pheochromocytomas contain significantly higher quantities of EM66, indicating that EM66 is a potential prognostic marker of chromaffin cell tumors.[17] Pheochromocytomas are usually benign but ~20% of these tumors are malignant, and to date the only evidence for malignancy is the occurrence of metastases, which are associated with a very poor survival rate.[18–20] The characterization of novel circulating markers is therefore crucial for the management of these tumors.

As a further step in the evaluation of EM66 as a marker for the diagnosis and prognosis of pheochromocytoma, the aim of the present study was to characterize this peptide in plasma and urine, and to compare its concentrations between healthy volunteers and patients with pheochromocytoma. The ability of pheochromocytes in primary culture to release EM66 was also investigated.

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***Correspondence to:** INSERM U413, European Institute for Peptide Research (IFRMP23), Laboratory of Cellular and Molecular Neuroendocrinology, University of Rouen, 76821 Mont-Saint-Aignan, France.
Fax: +33-235-146946. E-mail: hubert.vaudry@univ-rouen.fr

TABLE I – CLINICAL CHARACTERISTICS OF PATIENTS WITH PHEOCHROMOCYTOMA

	Patient		Pheochromocytoma			
	Sex	Age	Hereditary	Status	Site	Diameter (mm)
1[1,2,3]	M	65	No	Be	Right adrenal	50
2[1,2,3]	F	67	No	Be	Right adrenal	80
3[1,2]	M	61	No	Be	Left adrenal	58
4[1]	M	69	No	Be	Left adrenal	25
5[1]	M	69	No	Be	Right adrenal	70
6[1]	F	36	*SDHB*	Be	Ectopic	50
7[1]	F	62	MEN2	Be	Left adrenal	30
8[1]	F	26	No	Ma	Metastatic	60
9[3]	F	55	No	Be	Left adrenal	
10[3]	F	57	No	Be	Left adrenal	80
11[3]	F	53	No	Be	Left adrenal	32

[1]Preoperative plasma sample.–[2]Preoperative urine sample.–[3]Postoperative plasma sample.

Be, Benign; F, Female; M, Male; Ma, Malignant; MEN2, Multiple endocrine neoplasia type 2; *SDHB*, Succinate dehydrogenase subunit B gene (familial paraganglioma).

Material and methods

Blood and urinary samples

Blood samples were obtained from a group of 14 healthy volunteers (9 women and 5 men) and from 11 patients with pheochromocytoma (10 benign and 1 malignant) whose clinical characteristics are indicated in Table I. In 2 patients (cases no. 10 and 11, Table I), blood samples were collected at the clamping of the afferent vessels of the tumor by the surgeon (T_0) as described previously,[21] and then at 1, 2, 5, 10, 20, 30, 60 and 120 min after clamping during resection. For these patients, late samples were obtained 24 hr or 7 days after the operation. Urine samples were collected from a group of 20 controls (10 women and 10 men) and from 3 patients with pheochromocytomas (patients 1–3, Table I). Plasma and urine samples of patients were provided by a French endocrinology network for the collection of adrenal tumors (Réseau COMETE-2, PHRC AOM-02068), and by the Rouen and Lausanne University Hospital Centers. The patients gave written informed consent and the protocol of collection of the samples was approved by the regional bioethics committees (Comité Consultatif de Protection des Personnes dans la Recherche Biomédiale de Haute-Normandie et de Paris, France). After collection, plasma and urine samples were kept frozen at −80°C.

Production of recombinant EM66

A fusion protein containing the human EM66 peptide was produced in *Escherichia coli* as previously described.[16] Briefly, the cDNA sequence encoding the EM66 region in human SgII was amplified by PCR, inserted downstream of the maltose-binding protein (MBP) region of the plasmid pMAL-c2 (New England Biolabs, Beverly, MA) and expressed in bacteria. The cleavage of the affinity-purified fusion protein with factor Xa released EM66 with the native amino acid sequence.

Preparation of samples

Blood samples collected from healthy volunteers were either allowed to coagulate for 2 hr at room temperature (serum) or protected from coagulation with EDTA (plasma), centrifuged 20 min

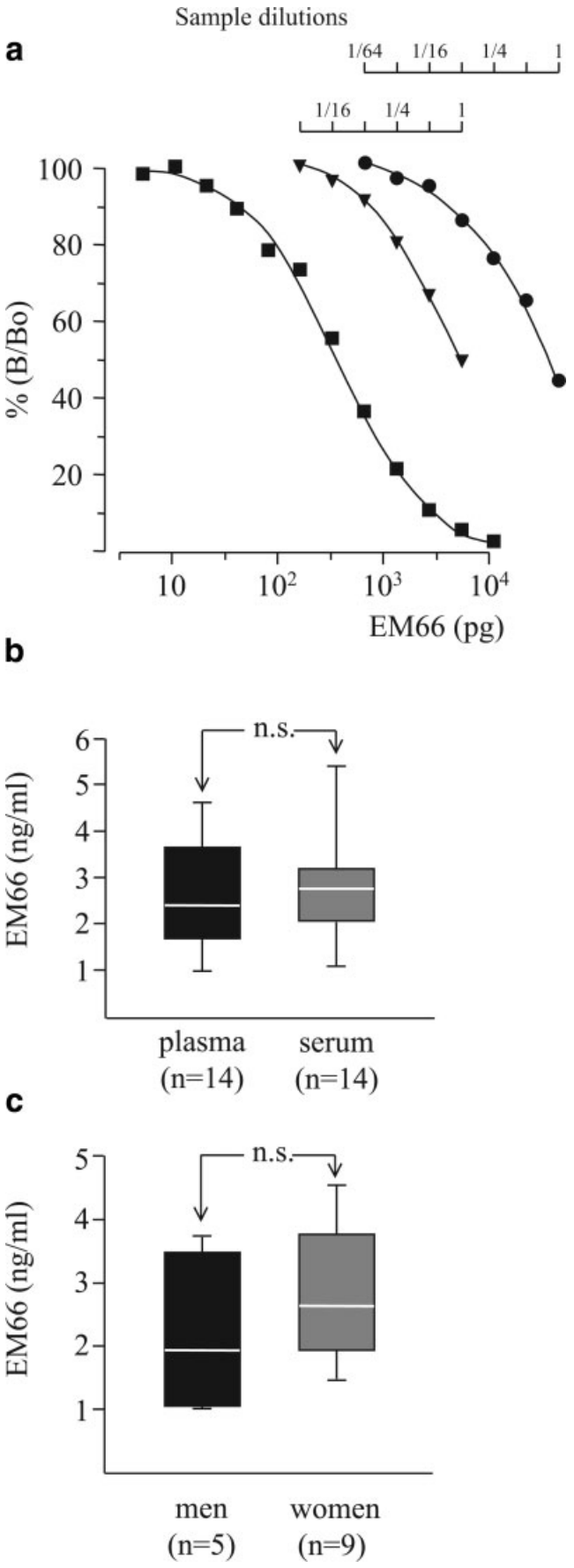

FIGURE 1 – EM66 levels in serum and plasma of healthy volunteers. (*a*) Semilogarithmic plots comparing competitive inhibition of antibody-bound ^{125}I-labeled EM66 by recombinant EM66 (■) and serial dilutions of plasma (▼) or serum (●) samples. (*b*) Box plots (with median, 25th–75th percentile and minimum–maximum values) showing EM66 levels in plasma and serum. (*c*) Comparison of plasma EM66 levels in men and women.

at 3,000*g* and filtered through a 0.22 μm membrane. The urine samples from healthy volunteers were directly filtered on Whatman paper after collection. Then, serum, plasma and urine samples were either kept at 4°C before prepurification for subsequent HPLC analysis or dried by vacuum centrifugation and kept at room temperature for further RIA quantification of EM66 concentrations. Plasma and 24 hr urine collection obtained from patients with pheochromocytoma were analyzed for concentrations of free or total metanephrines using a HPLC/coulometric detection technique according to the method previously described by Eisenhofer *et al*.[22] and Sawka *et al*.[23] For fractionated plasma free metanephrines, a normetanephrine value greater than 0.159 ng/ml or a metanephrine value greater than 0.076 ng/ml was considered positive, on the basis of a reference range established by our laboratory. For fractionated plasma total metanephrines, the upper reference limit was 4.93 ng/ml for normetanephrine and 1.13 ng/ml for metanephrine. For urinary normetanephrine and metanephrine, values greater than 2.91 and 1.02 μmol/24 hr, respectively, were considered positive.

Prepurification of serum and urinary samples

Each sample was loaded onto a Sep-Pak C_{18} cartridge (Waters Corp, St-Quentin en Yvelines, France) equilibrated with a solution of 0.1% trifluoroacetic acid (TFA) in water. Bound material was eluted from the cartridge with a solution of acetonitrile/water/TFA (59.9:40:0.1, vol/vol/vol), dried by vacuum centrifugation and kept at room temperature until chromatographic analysis.

HPLC analysis

Dried samples were reconstituted in 1 ml of 0.1% TFA in water, centrifuged at 21,000*g* (10 min; 4°C) and injected onto a 4.6 × 250 mm Vydac 218TP54 (C_{18}) column equilibrated with a solution of acetonitrile/water/TFA (9.9:90:0.1, vol/vol/vol) at a flow rate of 1 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 60% over 25 min using a linear gradient. HPLC standard consisted of 1 μg purified recombinant EM66. Fractions of 0.5 ml were collected, evaporated and kept dry until RIA.

Radioimmunoassay

The concentrations of EM66 in serum, plasma and urine samples were measured by RIA. Purified recombinant EM66 was iodinated by the chloramine-T method and separated from free iodine on Sep-Pak C_{18} cartridges using a gradient of acetonitrile (0–100%) in 0.1% TFA, as previously described.[16] The RIA was performed in veronal buffer (pH 7.4) supplemented with 0.4% bovine serum albumin (BSA, Roche Diagnostics, Mannheim, Germany) and 0.1% Triton X-100. The EM66 antiserum (code no. 736-1806), raised in rabbit against the recombinant fusion protein MBP-EM66 and used at a final dilution of 1:48,000, was incubated with 7,000 cpm of tracer/tube in the presence of graded concentrations of standard (purified EM66), serum, plasma or urine samples, or HPLC fractions. After a 2-day incubation at 4°C, the antibody-bound fraction was immunoprecipitated by the addition of 200 μl goat anti-rabbit γ-globulins (1:30), 200 μl normal rabbit serum (1:150) and 1 ml of 5% polyethyleneglycol 8000. After a 2 hr incubation at room temperature, the mixture was centrifuged (5,000*g*, 30 min, 4°C) and the pellet containing the bound fraction was counted on a gamma-counter (LKB, Wallack, Rockville, MD). The standard curve was set up with concentrations of EM66 ranging from 5 to 10,000 pg/tube. Assay precision was evaluated by adding to plasma samples EM66 at 4 different concentrations (2, 5, 10 and 20 ng/ml). The recovery for EM66 was 93–107%. Interassay coefficient of variation (CV) were 5.00–9.24% and intraassay CV were 0.74–1.98%.

Cell culture

After surgical removal, pheochromocytoma fragments were transferred into DMEM (Sigma-Aldrich), transported to the laboratory and rapidly processed as follows. The tumors were sliced into small pieces of about 3 mm^3 and then enzymatically digested (45 min, 37°C) in DMEM containing 0.1% collagenase (Serlabo, Bonneuil-sur-Marne, France) and 30 U/ml DNase I (Sigma-

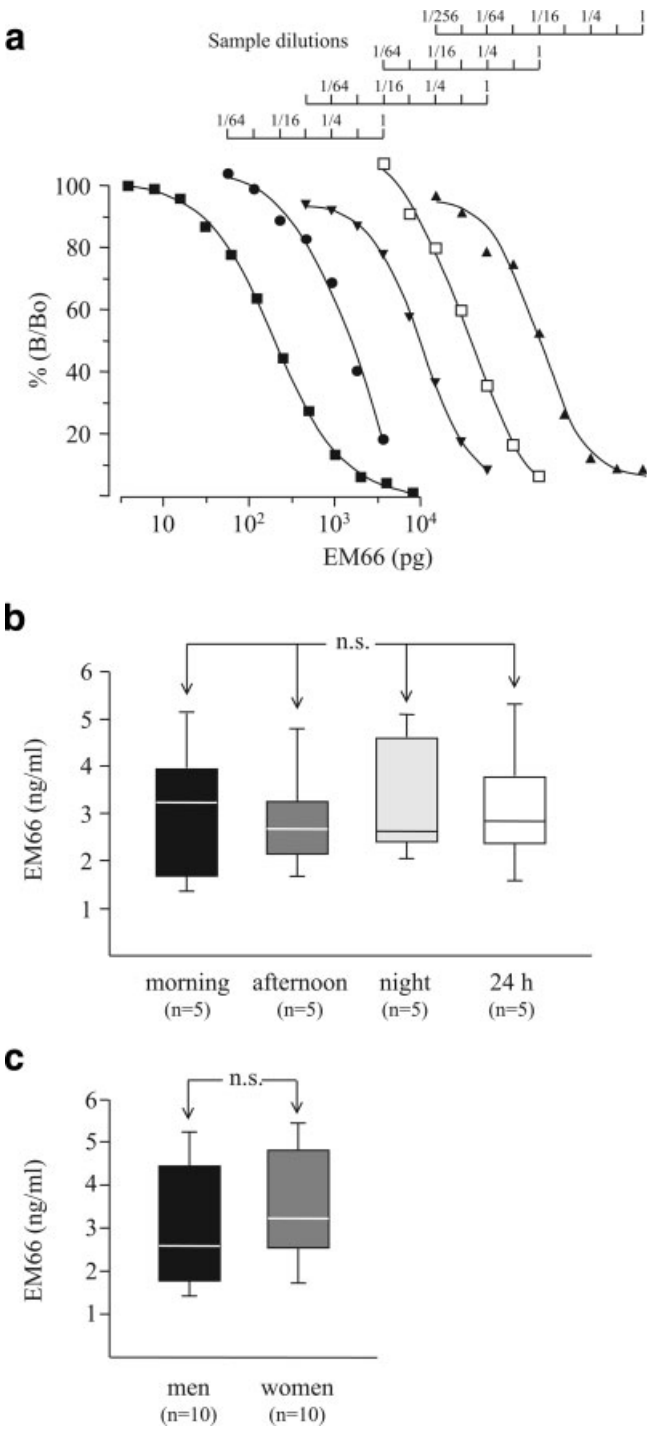

Figure 2 – EM66 levels in the urine of healthy volunteers. (*a*) Semilogarithmic plots comparing competitive inhibition of antibody-bound ^{125}I-labeled EM66 by recombinant EM66 (■) and serial dilutions of morning (●), afternoon (▼), night (□) or 24 hr period (▲) urine samples. (*b*) Box plots (with median, 25th–75th percentile and minimum–maximum values) showing EM66 concentrations collected punctually or during 24 hr. (*c*) Comparison of urine EM66 levels in men and women.

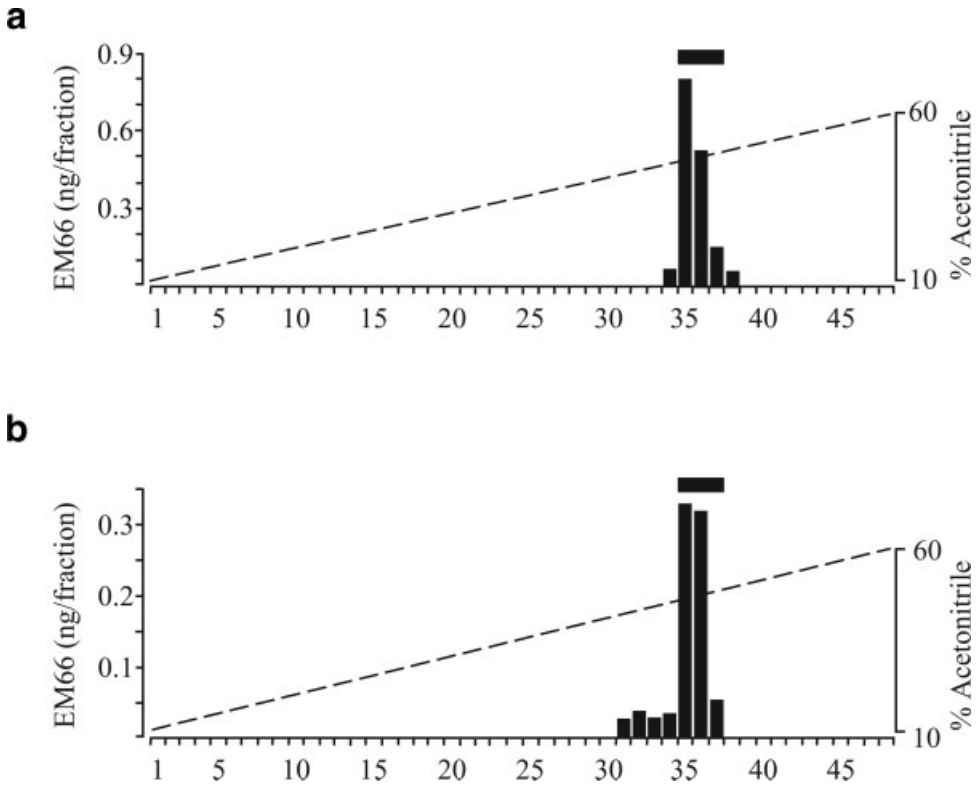

Figure 3 – Reversed-phase HPLC analysis of EM66 immunoreactivity in serum (*a*) and 24 hr urine (*b*) extracts from healthy volunteers. Samples were prepurified on Sep-Pak C_{18} cartridges and chromatographed onto a Vydac C_{18} column. Fractions were collected (0.5 ml/tube), dried and radioimmunoassayed for EM66. The bars above the peaks indicate the elution position of recombinant EM66 chromatographed the same day as the samples. The dashed lines show the concentration of acetonitrile in the eluting solvent.

Aldrich). The isolated cells were cultured in DMEM supplemented with 5% calf serum (Biowhittaker Europe, Verviers, Belgium), 10% horse serum (Invitrogen, Cergy Pontoise, France), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies, Cergy-Pontoise, France). Tumoral chromaffin cells were purified by diffential plating to remove adherent nonchromaffin cells, as described previously[24] and then plated at a density of 10^5 cells/500 μl per well. After 1 day, the culture medium was removed and pheochromocytes were incubated for 24 hr with fresh DMEM. The culture medium was then collected and immediately frozen at −20°C until RIA determination of EM66 immunoreactivity. For immunocytochemical experiments, cells were cultured on glass slides coated with poly-L-lysine (Sigma-Aldrich, Saint-Quentin Fallavier, France).

Normal adrenal glands were obtained from kidney transplant donors. All the donors were brain dead patients, whose relatives had accepted multiorgan procurement. Chromaffin cells were isolated by differential plating as previously described[25] and 95% pure chromaffin cell cultures were obtained. Normal chromaffin cells were incubated for 24 hr with fresh DMEM in the same conditions as pheochromocytes, and the supernatant was collected for RIA determination of EM66 immunoreactivity.

Immunocytochemical procedure

Slides with cultured pheochromocytes were dipped for 30 min in 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). After several rinses in PB, fixed cells were processed for indirect immunofluorescence. Pheochromocytes were incubated overnight at 4°C with the EM66 antiserum diluted 1:200 in PB containing 0.3% Triton X-100 and 1% BSA. Then, tumoral cells were rinsed in PB and incubated for 90 min at room temperature with fluorescein isothiocyanate-conjugated goat antirabbit γ-globulins (Caltag Laboratories, San Francisco, CA) diluted 1:100. Finally, cells were rinsed in PB, mounted with buffer/glycerol (1:1), coverslipped and examined using a confocal laser scanning microscope (CLSM TCS-SP2-AOBS, Leica) equipped with a fluorescence optical system DMRX-A2 and an argon (excitation wavelengths 458/476/488/514 nm) and 2 helium/neon (excitation wavelengths 543 and 633 nm, respectively) ion lasers. To verify the specificity of the immunoreaction the EM66 antiserum (diluted 1:200) was preincubated with purified EM66 (10^{-6} M).

Data analysis

Data are reported as median (25th–75th percentile), where the 25th–75th percentile represents the dispersion of the distribution. Several nonparametric statistical methods were used, Mann–Whitney *U* test, Kruskal–Wallis test and Wilcoxon signed rank test. The Spearman's nonparametric test was performed to analyze the correlation between plasma EM66 levels and tumor size. The half-life of EM66 was calculated by log-linear regression from the EM66 values obtained at the time of the clamping of blood vessels and at various times until reaching a residual flat concentration. Probability values less than 0.05 were considered significant. Data were analyzed with the Prism program (GraphPad Software, San Diego, CA).

Results

EM66 levels in serum, plasma and urine of healthy volunteers

Serial dilutions of serum and plasma samples generated displacement curves that were parallel to that obtained with recombinant EM66 (Fig. 1*a*). The plasma and serum EM66 concentrations in controls were, respectively, 2.4 (1.7–3.7) ng/ml ($n = 14$) and 2.8 (2.1–3.2) ng/ml ($n = 14$) and were not significantly different from each other (Fig. 1*b*). EM66 concentrations in men (1.9 (1.1–3.5) ng/ml) ($n = 5$) and women (2.6 (2.0–3.7) ng/ml) ($n = 9$) were not significantly different (Fig. 1*c*).

Urine samples were either collected punctually, *i.e.* in the morning (10–11 hr), in the afternoon (16–17 hr) and night (7–8 hr), or collected during a 24 hr period. Serial dilutions of urine samples generated displacement curves that were parallel to that obtained

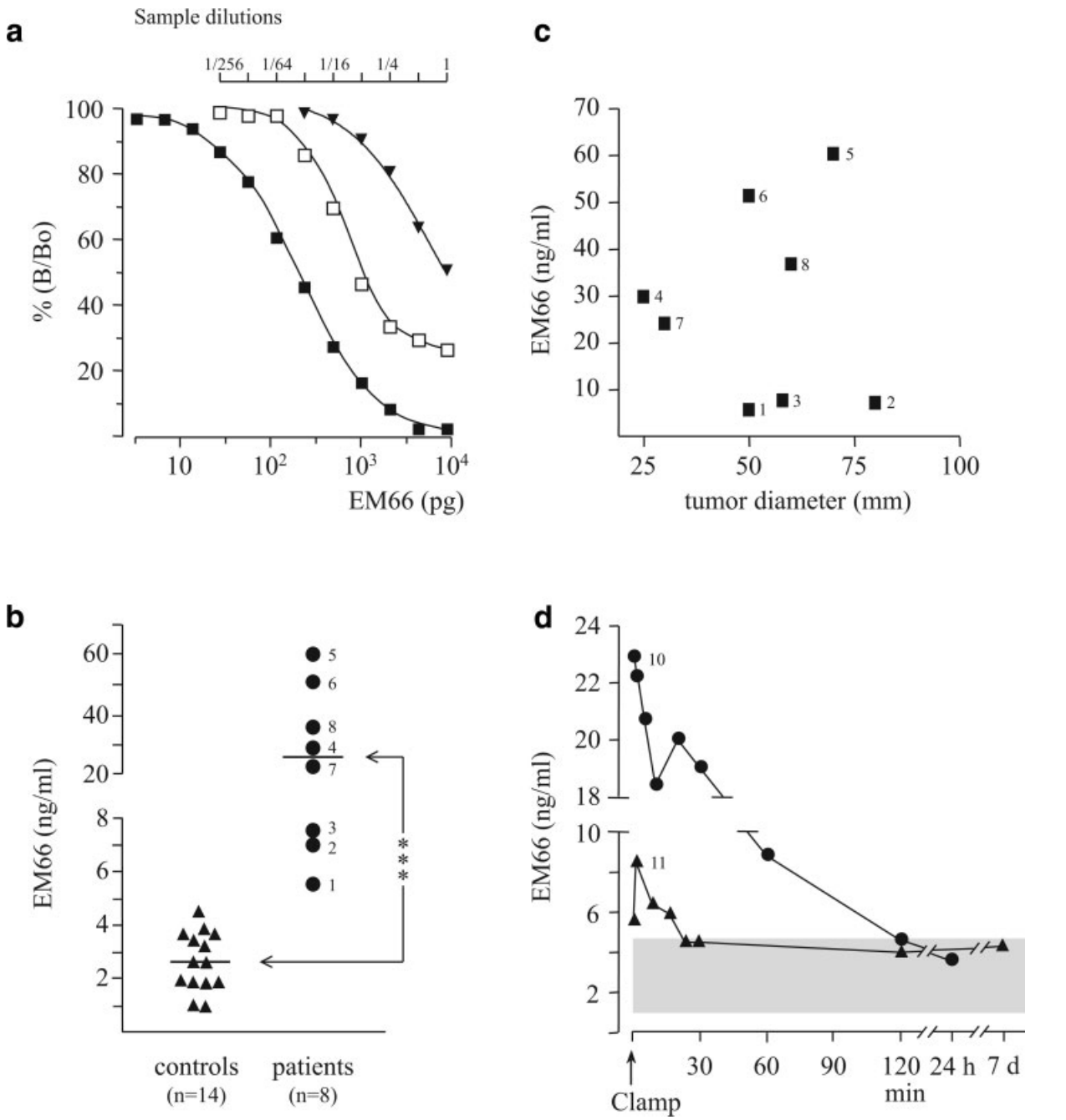

Figure 4 – EM66 levels in the plasma of patients with pheochromocytoma. (*a*) Semilogarithmic plots comparing competitive inhibition of antibody-bound ^{125}I-labeled EM66 by recombinant EM66 (■) and serial dilutions of preoperative (□) and postoperative (▼) plasma samples. (*b*) Scattergram of EM66 concentrations in plasma of healthy volunteers (▲) and patients with pheochromocytoma (●). The bars represent the median value for each group. ***, $p < 0.001$. (*c*) Scatter plot showing the distribution of preoperative plasma EM66 levels and tumor size (diameter) for each patient. (*d*) Time course of EM66 levels after the afferent vessels were clamped and the tumor was resected. The numbers refer to the patients in Table I. The shaded area represents the range of variation of plasma EM66 levels observed in healthy volunteers.

with recombinant EM66 (Fig. 2*a*). The concentrations of EM66 in urinary samples were 3.2 (1.7–4.0) ng/ml in the morning ($n = 5$), 2.7 (2.1–3.3) ng/ml in the afternoon ($n = 5$), 2.6 (2.4–4.6) ng/ml at night ($n = 5$) and 2.8 (2.4–3.8) ng/ml over 24 hr ($n = 5$) and were not significantly different from each other (Fig. 2*b*). The concentrations of EM66 in men (2.5 (1.8–4.4) ng/ml, $n = 10$) and in women (3.2 (2.5–4.8) ng/ml, $n = 10$) were not significantly different (Fig. 2*c*).

Characterization of EM66-immunoreactivity in serum and urine extracts

Biochemical characterization of EM66 immunoreactivity in the different samples was performed by HPLC analysis combined with RIA detection (Fig. 3). Recombinant EM66 eluted in fractions 35–37 (45–47% acetonitrile). In serum and urine extracts, a major immunoreactive peak was resolved that coeluted with recombinant EM66 (Figs. 3*a* and 3*b*).

TABLE II – SERUM, PLASMA AND URINE CONCENTRATIONS OF EM66 IN HEALTHY VOLUNTEERS AND PATIENTS WITH PHEOCHROMOCYTOMA

	Median	Minimum	25%	75%	Maximum
Healthy volunteers					
Blood ($n = 14$)	2.6 (335)	1.1 (129)	1.9 (239)	3.7 (469)	4.5 (576)
Plasma ($n = 14$)	2.4 (309)	1.0 (125)	1.7 (217)	3.7 (467)	4.6 (586)
Serum ($n = 14$)	2.8 (355)	1.1 (133)	2.1 (271)	3.2 (407)	5.4 (683)
Men ($n = 5$)	1.9 (246)	1.1 (129)	1.1 (138)	3.5 (445)	3.7 (469)
Women ($n = 9$)	2.6 (335)	1.5 (192)	2.0 (250)	3.7 (477)	4.5 (576)
Urine ($n = 20$)	2.9 (368)	1.4 (174)	1.9 (236)	4.6 (585)	5.4 (679)
Morning ($n = 5$)	3.2 (411)	1.4 (174)	1.7 (213)	4.0 (503)	5.1 (552)
Afternoon ($n = 5$)	2.7 (340)	1.7 (213)	2.1 (272)	3.3 (418)	4.8 (612)
Night ($n = 5$)	2.6 (335)	2.1 (261)	2.4 (302)	4.6 (585)	5.1 (647)
24 hr ($n = 5$)	2.8 (361)	1.6 (202)	2.4 (300)	3.8 (481)	5.4 (679)
Men ($n = 10$)	2.5 (320)	1.7 (213)	1.8 (226)	4.4 (564)	5.4 (679)
Women ($n = 10$)	3.2 (401)	1.4 (174)	2.5 (321)	4.8 (612)	5.1 (652)
Patients					
Plasma					
Preoperative ($n = 8$)	26.9 (3,409)	5.6 (710)	7.3 (929)	44.0 (5,576)	60.3 (7,650)
Postoperative					
<12 days ($n = 2$)	12.5 (1,590)	8.6 (1,086)			16.5 (2,094)
>10 months ($n = 2$)	1.9 (237)	1.1 (133)			2.7 (341)
Urine					
Preoperative ($n = 3$)	3.2 (403)	1.8 (230)	2.2 (273)	3.9 (499)	4.2 (531)

Results are expressed as median, 25th-75th percentile and minimum and maximum values. Values are given in ng/ml and pM in parentheses. For healthy volunteers, blood values correspond to the mean of plasma and serum values measured in the same individual.

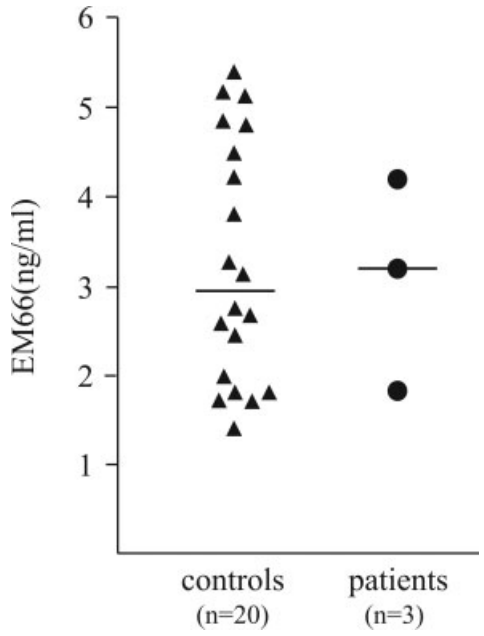

FIGURE 5 – Scattergram of EM66 concentrations in urine samples of healthy volunteers (▲) and patients with pheochromocytoma (●). The bars represent the median value for each group.

EM66 levels in the plasma and urine of patients with pheochromocytoma

Serial dilutions of preoperative and postoperative plasma extracts generated displacement curves that were parallel to that obtained with recombinant EM66 (Fig. 4*a*). In a group of 8 patients with chromaffin cell tumors (7 benign and 1 malignant; Table I), preoperative plasma EM66 ranged from 5.6 to 60.3 ng/ml with a median value 10-fold higher than that measured in the group of healthy volunteers [26.9 (7.3–44) *vs.* 2.6 (1.9–3.7) ng/ml, $p < 0.001$] (Fig. 4*b*). The patient with a malignant pheochromocytoma (case no. 8 in Table I) had a plasma EM66 level of 36.7 ng/ml (Fig. 4*b*). No correlation was found between preoperative plasma EM66 levels and tumor size of the patients (Spearman correlation coefficient = 0.048; $p = 0.934$) (Fig. 4*c*). Once the afferent vessels were clamped and the tumor was resected, EM66 concentrations returned to basal level within 23 or 120 min, depending on the concentration of the peptide at the time of clamping (patients 11 and 10 in Table I, respectively) (Fig. 4*d*). The mean value for the half-life of EM66 was 38.1 min with individual estimates ranging from 25.7 (patient 11) to 50.6 min (patient 10). In 2 patients (1 and 9 in Table I), 12 days after surgical removal of the tumor, EM66 concentrations decreased but were still elevated compared to the control group (12.5 *vs.* 2.6 ng/ml; Table II). The follow-up of the patients showed that EM66 was in the normal range after few months (1.9 *vs.* 2.6 ng/ml; Table II) (patients 1 and 2 in Table I).

In 3 patients with benign pheochromocytoma (patients 1–3 in Table I), the preoperative urinary EM66 levels were in the same range as those of normal subjects [3.2 (2.2–3.9) *vs.* 2.9 (1.9–4.6) ng/ml, respectively] (Fig. 5). EM66 concentrations measured in serum, plasma and urine extracts of healthy volunteers or pheochromocytoma patients are summarized in Table II.

Metanephrine levels

Preoperative plasma and/or urinary metanephrine concentrations were measured during the hospitalization period of the patients with pheochromocytoma. In our series, several patients had urinary normetanephrine and metanephrine levels under the upper reference limit, the sensitivity of these 2 tests was, respectively, 50 and 62.5% (Figs. 6*a* and 6*b*). Similary, plasma total normetanephrine and metanephrine, as well as plasma free normetanephrine and metanephrine measurements, generated false-negative results, the sensitivity of these tests was, respectively, 60, 60, 60 and 20% (Figs. 6*c*–6*f*). For one patient (patient 7 in Table I), 4 out of the 6 tests were negative. Plasma and urinary metanephrine concentrations measured in patients with pheochromocytoma are summarized in Table III.

EM66 in cultured pheochromocytes and chromaffin cells

Immunohistochemical labeling with EM66 antibodies produced intense staining of the cytoplasm of pheochromocytes in primary culture (Fig. 7*a*). Preabsorption of the EM66 antiserum

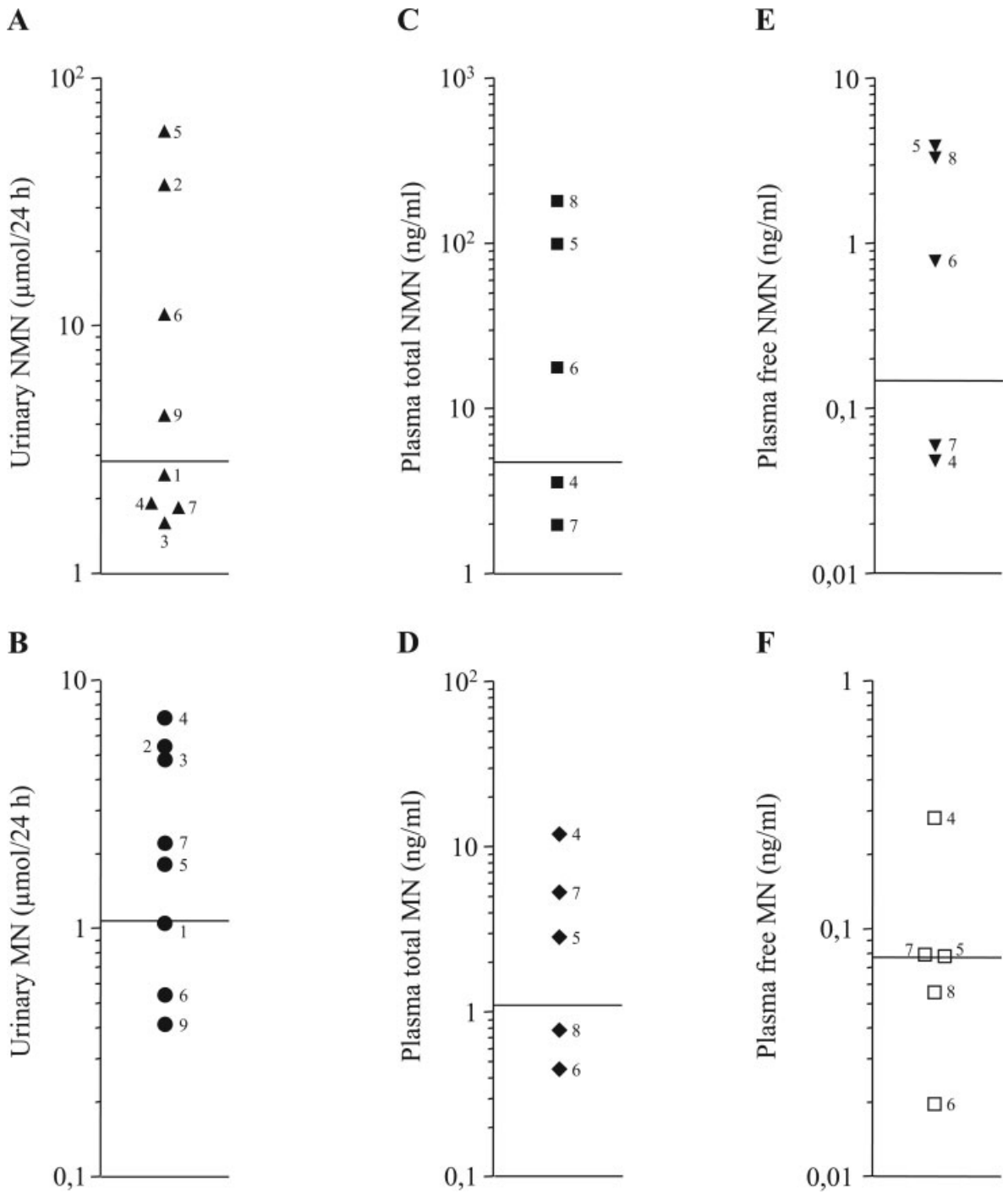

Figure 6 – Urine and plasma catecholamine metabolite levels in patients with pheochromocytoma. (*a*) Urine normetanephrine (▲); (*b*) urine metanephrine (●); (*c*) plasma total normetanephrine (■); (*d*) plasma total metanephrine (◆); (*e*) plasma free normetanephrine (▼); (*f*) plasma free metanephrine (□). The bars represent the generally admitted upper reference limit in normal subjects. The numbers refer to the patients in Table I. Free metanephrines correspond to unconjugated metabolites and total metanephrines refer to free metanephrines plus sulfate-conjugated metanephrines. NMN, normetanephrine; MN, metanephrine.

with 10^{-6} M recombinant EM66 totally abolished the immunoreaction (data not shown). EM66 secretion in the culture medium of benign pheochromocytes was 2.9 ± 0.3 ng/10^4 cells per 24 hr (patient 1) and 1.6 ± 0.2 ng/10^4 cells per 24 hr (patient 2) (Fig. 7*b*). Cultured normal chromaffin cells released 20–35-fold less EM66 (0.082 ± 0.005 ng/10^4 cells per 24 hr) compared to pheochromocytes from patients 1 and 2, respectively.

Discussion

We have recently described the presence of the SgII-derived peptide EM66 in the rat pituitary and in the rat and human adrenal glands.[15,16] Here, we show that substantial amounts of EM66 (in the nanomolar range) also occur in the plasma/serum of healthy volunteers. HPLC analysis of serum extracts combined with RIA

detection resolved a single immunoreactive peak exhibiting the same retention time as the recombinant peptide, suggesting that authentic EM66 is released from neuroendocrine tissues into the circulation. The fact that similar concentrations of EM66 were measured in serum and plasma indicates that the mode of collection of blood samples should not affect the determination of circulating EM66 levels. We found similar high levels of EM66 in urine and plasma samples (2.9 *vs*. 2.6 ng/ml, respectively), indicating that EM66, as SN,[5] is efficiently filtered and excreted by the kidney. Moreover, HPLC analysis demonstrated that, in urine as in serum, EM66 immunoreactivity corresponded to the intact 66-amino acid form of the peptide. Comparison of urine EM66 concentrations collected punctually during different periods of the day did not reveal any circadian variations of peptide excretion or any differences between women and men.

Because EM66 is present in tumoral chromaffin cells,[17] this peptide could also be released in the circulation of patients with pheochromocytoma. Therefore, we characterized EM66 in the plasma of such patients and compared its concentrations with those of healthy volunteers. In a group of 8 patients bearing the tumor, EM66 was readily detectable in the plasma, and preoperative EM66 levels were 10-fold higher compared to control subjects, suggesting that tumoral cells produce and release higher amounts of EM66. To determine whether the elevated concentration of EM66 in the plasma of pheochromocytoma patients was attributable to secretion of the peptide from the tumor, we performed cultures of nontumoral and tumoral chromaffin cells. The data showed that pheochromocytes in primary culture had the ability to release 20–35-fold higher amounts of the peptide into the medium than did healthy chromaffin cells, suggesting that, *in vivo*, EM66 is actually released from the tumor into the circulation of the patients. In agreement with this hypothesis, we observed that, during surgical removal of the pheochromocytoma, plasma EM66 concentrations declined to values similar to those measured in control subjects within 23–120 min, with a mean half-life estimated to 38 min. However, in 2 patients, 12 days after surgical removal of the tumor, EM66 concentrations decreased but were still elevated compared to those in the control group. The remaining high levels of EM66 after resection may be attributed to the stress generated by the surgical operation. In support of this hypothesis, Baudin *et al*.[8] have shown that major stress associated with catecholamine secretion also increased CgA serum level. Preoperative treatment of patients with pheochromocytoma is based on the use of α- and β-blockers, and it is usually observed that the cessation of this treatment is responsible for a flare-up of the sympathetic nerve system, which in turn leads to an elevation of catecholamine levels that return to normal range only within 1 week.[21] Therefore, Cgs that are costored and coreleased with catecholamines may present recurrent elevated levels soon after surgical resection. Overall, our data indicate that the measurement of EM66 in plasma may represent a novel highly sensitive clinical tool for the diagnosis and follow-up of chromaffin cell tumors. Because pheochromocytoma is a rare tumor, a relatively small number of patients could be followed for EM66 measurement after surgery. Larger number of preoperative and postoperative plasma samples should be explored in the future to substantiate this observation.

Currently, CgA is the only granin routinely used as a biological marker of pheochromocytomas.[26–28] Indeed, CgA is a general marker for a variety of neuroendocrine tumors, including mid- and forgut carcinoids, pancreatic islet-cell tumors and bronchial carcinoids.[6,8] However, a recent study by Stridsberg *et al*.[29] comparing 3 commercial kits for plasma CgA measurements in various neuroendocrine tumors has revealed that, depending on the kit used, the sensitivity of the CgA test varied between 67 and 93%. Thus, plasma measurement of CgA may produce false-negative test results in the diagnosis of neuroendocrine tumors. In addition, it has been shown that plasma CgA determination may also lead to false-positive results in certain circumstances such as type A gastritis,[30] decreased renal function[31] and treatment with proton pump inhibitors.[32] Other biochemical tests used for the diagnosis of pheochromocytoma are the measurement of plasma and urinary catecholamines (epinephrine and norepinephrine) and catecholamine metabolites (metanephrine and normetanephrine).[33] These tests also have limitations and some are better than others for confirming or excluding pheochromocytoma. For instance, some chromaffin cell tumors do not secrete enough catecholamines to produce positive results or many pheochromocytomas release catecholamines episodically so that, between episodes, levels of the amines are in the normal range generating false-negative results.[34] On the other hand, metanephrine production is independent of catecholamine release[22] and it is now well established that measurement of plasma or urinary metanephrines provides better diagnostic results than catecholamines do. Several authors proposed that measurement of plasma free (sulfate-unconjugated) metanephrines is the test of choice for the diagnosis of pheochromocytomas[33,35,36] while others argue that measurement of plasma total

TABLE III – CONCENTRATIONS OF PLASMA AND URINARY METANEPHRINES IN PATIENTS WITH PHEOCHROMOCYTOMA

	Upper reference limit	Preoperative
Plasma ($n = 5$)		
Free metanephrines (ng/ml)		
Normetanephrine	0.16	0.82 (0.05–3.7)
Metanephrine	0.08	0.08 (0.02–0.3)
Total metanephrines (ng/ml)		
Normetanephrine	4.93	17.8 (2–178)
Metanephrine	1.13	2.9 (0.46–11.8)
Urine ($n = 8$)		
Metanephrines (μmol/24 hr)		
Normetanephrine	2.91	3.4 (1.6–62)
Metanephrine	1.02	1.9 (0.4–6.6)

Values are expressed as median; the minimum and the maximum values are given in parentheses.

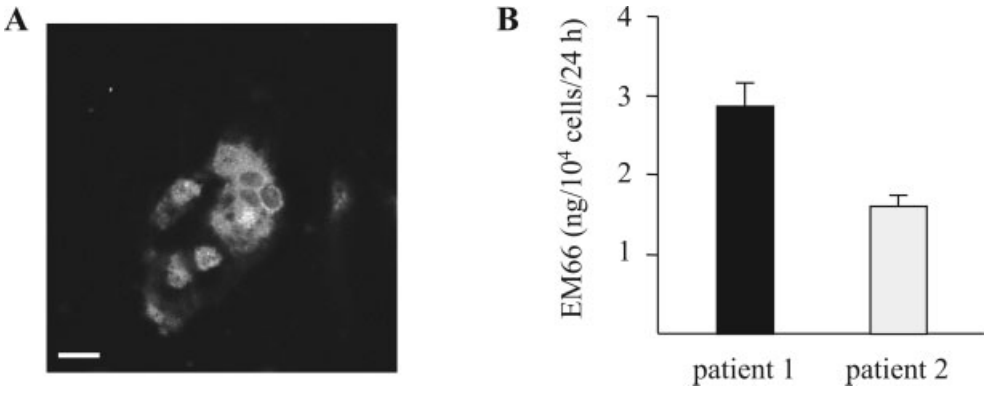

FIGURE 7 – (*a*) EM66-immunocytochemical labeling of pheochromocytes in primary culture. An intense signal is observed in the cytoplasm of isolated or clustered tumoral cells. Bar, 12.5 μm. (*b*) Levels of EM66 released from cultured pheochromocytes. Histograms showing mean ± SEM of EM66 concentrations in incubation media from pheochromocytoma cells originating from patients 1 and 2.

metanephrines (free + sulfate-conjugated) offers the highest sensitivity.[21] In our study, whatever the metanephrine test used, false-negative results were obtained. For instance, catecholamine metabolite measurements in patient no. 7 led to 4 false-negative results over 6 tests. These observations indicate that, while combined measurement of various forms of metanephrines offers a high diagnostic sensivity, this biochemical test alone is obviously not sufficient for the screening of pheochromocytomas. Besides false-negative results, false-positive results can also be generated when one measures metanephrines produced as a result of deficiency or pharmacological inhibition of monoamine oxidase (which leads to increased urinary deconjugated and plasma free metanephrines), or by medications such as tricyclic antidepressants (which account for up to 45% of false-positive elevation of plasma or urinary norepinephrine and normetanephrine).[36,37]

We have previously shown that a low EM66 concentration in pheochromocytoma tissues is indicative of a malignant differentiation of the tumor.[17] Because only one malignant pheochromocytoma was included in the present study, no conclusion can be drawn concerning the value of EM66 as a plasma prognostic marker. Further studies will be required to compare the concentration of EM66 in patients with benign *vs*. malignant tumors, and to determine whether, in addition to its usefulness for the diagnosis and follow-up of pheochromocytoma, EM66 could also be used to evaluate the outcome of the disease.

EM66 is also detected in urine of healthy volunteers and patients with pheochromocytoma. An elevation of preoperative EM66 concentrations in urine samples, as in plasma, could also be expected. Surprisingly, in the 3 patients examined, urine EM66 levels were in the range of those in the controls. It should be noted, however, that elevation of the levels of CgA, CgB and SgII fragments in urine of patients with neuroendocrine tumors was associated with renal tubular dysfunction.[38,39] Moreover, several studies indicated that the presence of CgA in the urine of patients with neuroendocrine neoplasms resulted from pharmacological treatment, renal dysfunction or structural abnormalities.[40,41] In accordance, our data indicate that determination of EM66 levels for the management of pheochromocytoma should be performed in plasma and that urine EM66 has no predictive value for the occurrence of this tumor.

In conclusion, we have demonstrated that the SgII-derived peptide EM66 occurs in biological fluids and constitutes a highly sensitive marker for the diagnosis and follow-up of pheochromocytomas. Currently, false-negative and false-positive results using catecholamine and catecholamine metabolites assay tests remain a problem, leading to cost-effective and time-consuming additional testing and imaging examinations.[23,42] A recent study reported that, among 80 patients who underwent ^{123}I-meta-iodobenzylguanidine scintigraphy in internal medicine for pheochromocytoma suspicion, only 18 (22.5%) actually had a tumor.[43] While our results need to be substantiated in a larger group of patients, we propose that the measurement of plasma EM66 level should be combined with the determination of other biological markers, such as metanephrines and CgA, for the management of pheochromocytoma.

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