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RESEARCH ARTICLE

Development of an immunoassay for the derived-peptide of chromogranin A, Vasostatin-I (1-76): assessment of severity in patients with sepsis

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Abstract

Context: Proteolytic fragments of chromogranin A (CgA) including the CgA 1-76 fragment (called vasostatin-I [VS-I]) could be a useful biomarker of sepsis, but there is no available immunoassay.

Methods: A sandwich ELISA for VS-I was developed, and plasma VS-I was measured in 30 healthy controls and 60 critically ill patients with sepsis.

Results: The ELISA showed intra- and inter-assay coefficients of variations (CVs) below 4 and 9%. Plasma VS-I was significantly increased compared with controls in patients with sepsis, severe sepsis, and sepsis shock ($p < 0.0001$). Receiver operating curve (ROC) analyses indicated that plasma VS-I was more sensitive and specific than plasma CgA to diagnose sepsis and to assess its severity.

Conclusions: The measurements of plasma VS-I with this new ELISA may be useful for the clinical investigation of patients with sepsis.

Keywords: Biomarker, chromogranin A, sepsis, vasostatin

Introduction

Sepsis is a systemic inflammatory response to infection. Various stages of gravity are described from sepsis to septic shock, which can lead to organ failure and eventually to death. Detection and prognosis of sepsis severity is crucial in the early stage of the disease, as any delay in giving proper treatments leads to poorer outcome (Pierrakos & Vincent, 2010). Evaluation of sepsis is complicated by the highly variable and nonspecific nature of the signs and symptoms (Lever & Mackenzie, 2007).

Besides conventional clinical parameters and the gram staining and culturing tests to confirm microbial infection, few biomarkers are used to diagnose sepsis and predict its outcome. C-reactive protein (CRP) is commonly utilized as an inflammation marker, but it is not specific of the infection and its value has been challenged (Pierrakos & Vincent, 2010). Procalcitonin is used as a marker of infection (Becker et al., 2010) but has limited prognostic value in nonselected patients with fever (Ivády et al., 2011). Potential biomarkers have been proposed including interleukin 6 (IL-6), tumor necrosis factor α, and triggering receptor expressed on myeloid cells 1 (Pierrakos & Vincent, 2010; Ivády et al., 2011), but clinical data are still very scarce. Among these new candidates, chromogranin A (CgA) has shown to predict survival independently of other risk factors in critically ill patients (Zhang et al., 2008).

Human CgA is a 439 amino acids glycoprotein stored in the secretory granules of nervous, endocrine, and

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immune cells (Helle et al., 2007). Commonly used as a neuroendocrine tumor marker (Conlon, 2010), it is also produced by other tissues such as the heart (Ceconi et al., 2002; Glattard et al., 2006; Pieroni et al., 2007). CgA is known to be processed through its numerous dibasic sites by the prohormone convertases PC1/3 and PC2, costored in the granules with CgA (Conlon, 2010). This intracellular and extracellular processing leads to several biologically CgA-derived peptides (Metz-Boutigue et al., 1993). Among them, the N-terminal fragment CgA 1-76 (called vasostatin-I [VS-I]), represents one of the predominant resulting fragment (Metz-Boutigue et al., 1993) and has shown to have different biological activities. It enhances the endothelial barrier function (Belloni et al., 2007), displays antimicrobial activities (Lugardon et al., 2000, 2001), and regulates heart contractility (Helle, 2010).

Currently, there is no assay available for measuring circulating VS-I. The aim of this study was to develop an ELISA for human VS-I and analyze its levels in patients with sepsis.

Materials and methods

Patients

We measured VS-I and CgA with ELISA in 60 consecutive critically ill patients at admission (mean age 60 years) and 30 healthy controls (mean age 42 years, 65% male). Patients were considered septic only if an infectious agent could be identified in any biological sample from a focus of infection, and they were further classified into septic conditions according to standard definitions of sepsis, severe sepsis, and septic shock (Bone et al., 1992). A patient was considered to be in shock if mean arterial pressure was less than 70 mm Hg or the systolic blood pressure was less than 100 mm Hg despite the fact that an adequate amount of fluids (at least 1000 mL of crystalloids or 500 mL of colloids) had been administered (unless there was an elevation in the central venous pressure to >12 mm Hg), and if there were signs of tissue hypoperfusion (e.g., altered mental state, mottled skin, urine output of <0.5 mL per kg of body weight for 1 h, or a serum lactate level >2 mmol per L. In case of shock, patients were infused with norepinephrine to maintain mean arterial pressure above 65 mm Hg. The diagnosis of sepsis and its gravity was assessed at the admission.

All blood measurements were performed on lithium-heparin plasma samples stored at –80°C until analysis.

The studies were approved by the local ethics committee, and written informed consent was obtained from each participant.

VS-1 (1-76) assay description

Reagents

96-well microtiter plates Nunc Immobilizer™, streptavidin was used according to the manufacturer's recommendation. Bovine serum albumin (BSA) free protease, goat anti-rabbit lgG-horseradish peroxidase conjugate (GAR-HRP), normal goat serum (NGS), *N,N*-dimethyl formamide anhydrous (DMF), aprotinin, and tween 20 were from Sigma Aldrich (St. Louis, MO). Biotin-NHS was from Calbiochem (Darmstadt, Germany). Proclin was from Supelco (Bellefonte, PA). EDTA and H_2SO_4 were obtained from Merck (Darmstadt, Germany). StabilZyme® HRP Conjugate Stabilizer was from Surmodics (Eden Prairie, MN). Chromogenic substrate TMB (3, 3′, 5, 5′-tetramethylbenzidine) was from KPL (Gaithersburg, MD).

The mouse monoclonal antibody (m5A8) raised against the CgA 53-57 epitope has been described previously (Corti et al., 1997; Roatta et al., 2011). The polyclonal antibody raised against the C-terminal residues 70-76 of VS-I (unable to cross-react with CgA) was raised in rabbits by immunization with the peptide CgA 70-76 coupled to keyhole limpet hemocyanine. Human recombinant VS-I and CgA 1-78 were prepared essentially as described previously (Corti et al., 1997).

Assay procedure

One hundred µL of biotinylated capture m5A8 antibody was added into streptavidin-coated microtiter plates and incubated at room temperature (RT) for 1 h under agitation. After washing, 100 µL of calibrators or samples diluted in 50 mM sodium phosphate buffer pH 7.4 containing 0.5% BSA, 0.1% proclin, 5 mM EDTA, and 0.001% aprotinin were added in duplicate and incubated for 1.5 h at RT. After washing, the captured VS-I molecules were detected by the polyclonal antibody (100 µL) diluted 1:5000 in 50 mM sodium phosphate buffer containing 0.5% BSA and 1% NGS. After incubation (1 h, RT), the unfixed reagents were eliminated, and 100 µL of GAR-HRP was added and left to incubate for 30 min at RT. After washing, 100 µL of HRP substrate (TMB) was added. The chromogenic reaction was stopped after 15 min with 100 µL of 1N H_2SO_4, and the absorbance of each sample was then determined at 450 nm and 620 nm (reference wavelength).

CgA assay

The CgA kit (Chromo@, Cisbio Bioassays, Codolet, France) is a sandwich ELISA using two monoclonal antibodies recognizing the midregion of CgA within the 145–234 sequence. The intra- and inter-assay coefficients of variation (CV) are lower than 6 and 12%, respectively.

Statistical analysis

Associations between VS-I, CgA, CRP, lactates, and leucocyte count were analyzed by Spearman rank correlation. Comparisons of continuous variables between patient groups were analyzed by the nonparametric Kruskal–Wallis and Mann–Witney tests. The diagnostic value of VS-1 and other biological variables to identify patients with severe sepsis or sepsis shock from healthy controls or patients with sepsis were assessed by constructing the receiver operating curve. The area under ROC and 95% confidence interval (CI) was also calculated for each variable.

Results

VS-I ELISA analytical performances

The intra-assay CV determined by measuring VS-I in the plasma of three patients with three different levels of VS-I (low, medium, and high concentrations) were <4%. The inter-assay CVs determined using two different plasma samples in 15 independent runs were <9%. The analytical limit of detection defined as the concentration which is two SD (standard deviation) above standard 0 was 0.22 ng/mL. The lower limit of quantification, defined as the lowest concentration that can be measured with an imprecision of 20%, was determined from the measurements of 32 samples and found to be 0.34 ng/mL.

The analytical recovery, as measured with plasma samples spiked with calibrators 1 to 5 (0.625; 1.25; 2.5; 5, and 10 ng/mL) ranged between 89 and 93%. Three different plasma samples were serially diluted, and the recovery of the dilution test was between 97% and 107%.

No significant loss of immunoreactivity was observed up to three freeze-thaw cycles of two plasma samples.

Full-length human recombinant CgA and CgA 1-78 were not detected by the VS-I ELISA up to a concentration of 23.8 and 11.7 nM, respectively.

Patient demographics at inclusion

In all patients with sepsis, severe sepsis and sepsis chock the infectious agent could be identified. The vast majority (90%) of infections was from bacterial origin. Other infectious agents include *Candida albicans* ($n = 2$) and *Plasmodium falciparum* ($n = 4$). There was a large diversity in the type of bacteria including by prevalence order *Streptococcus pneumonia* (20%), *E. Coli* (18.3%), *Staphylococcus aureus* (13.3%), *Pseudomonas aeruginosa* (8.4%), *Streptococcus B* (5%), *Klebsiella pneumonia* (5%), and *Haemophilus influenza* (5%). For the rest (15% of subjects), there was only one patient per type of bacteria which included *Streptococcus oralis*, *Streptococcus beta haemolyticus*, *Streptococcus agalactiae*, *Staphylococcus epidermis*, *Legionella pneumophila*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Enterobacter aerogeus*, and *Acinetobacter baumanii*.

At admission, 19 out of the 60 patients with sepsis had no comorbidities. The other patients had chronic obstructive lung disease ($n = 7$), ischemic heart disease ($n = 5$), hypertension ($n = 4$), stroke ($n = 4$), diabetes ($n = 4$), chronic renal failure ($n = 3$), cancer ($n = 3$), acute leukemia ($n = 2$), alcoholism ($n = 2$), drug addiction ($n = 2$), lymphoma ($n = 1$), status epilepticus ($n = 2$), myxoedema ($n = 1$), and valvular heart disease ($n = 1$). The distribution of comorbidities was similar between the three groups of patients with sepsis.

The average age was similar in patients with sepsis, severe sepsis, and sepsis chock (Table 1). There was a larger proportion of male subjects in the group of patients with sepsis only compared with the two other groups, although the difference did not reach statistical significance (Table 1).

Plasma VS-I levels in patients with sepsis and correlations with other biological markers

In the whole population of patients with sepsis, VS-I did not correlate with age ($p = 0.35$) and levels were similar in women and men ($p = 0.35$). There was no association between VS-I and the type of comorbidities at admission ($p = 0.25$). The association of VS-I with CgA was high in healthy controls ($r = 0.70$, $p < 0.0001$), but modest in patients with sepsis ($r = 0.53$, $p < 0.0001$) (Figure 1).

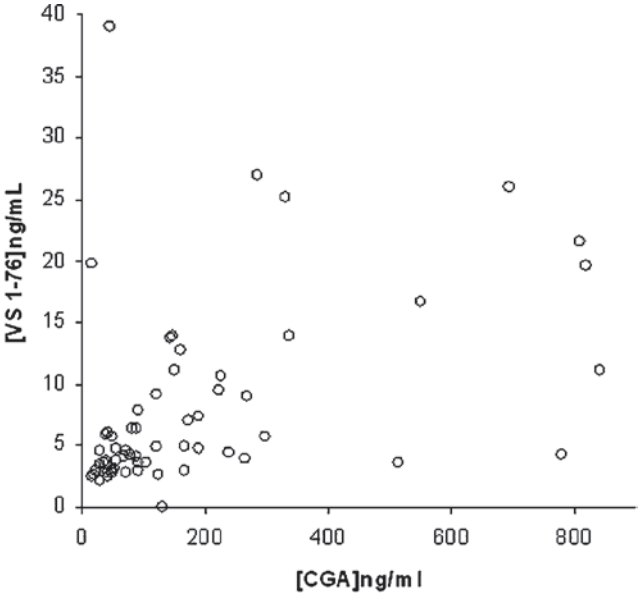

Figure 1. Correlation between plasma vasostatin-I and chromogranin A in 60 patients with sepsis. The spearman r value was 0.53 ($p < 0.0001$).

Table 1. Demographical and biochemistry data in patients with sepsis, severe sepsis, and sepsis shock.

Variable	Sepsis ($n = 20$)	Severe sepsis ($n = 20$)	Sepsis shock ($n = 20$)	p value
Gender (% male)	81%	50%	58%	0.39
Age (year)	57 ± 19 (52)	61 ± 17 (62)	64 ± 17 (62)	0.43
VS-I (ng/mL)	3.79 ± 2.03 (3.6)	11.41 ± 9.75 (8.02)**	9.15 ± 6.86 (6.2)**	0.0002
CgA (ng/mL)	50.2 ±188 (70.8)	126 ±192 (136)	215 ± 267 (93)	0.20
Lactates (mmol/L)	2.49 ± 1.73 (2.00)	3.73 ± 3.86 (2.55)*	5.04 ± 3.74 (4.50)*	0.035
CRP (mg/L)	106 ± 106 (72.5)	149 ± 77 (175)	198 ±124 (242)	0.12
Leucocyte count ($10^3/mm^3$)	13.7 ± 9.7 (11.9)	12.8 ± 9.6 (11.0)	19.1 ± 23.5 (13.6)	0.68

Results are shown as mean ± SD (median).
CgA, chromogranin A; CRP, C-reactive protein; VS-I, vasostatin-I.
*$p < 0.05$, **$p < 0.0005$ versus sepsis

In patients with sepsis, VS-I did not correlate with CRP ($p = 0.79$), lactates ($p = 0.37$), or leucocyte count ($p = 0.47$).

As shown on Figure 2, patients with sepsis, severe sepsis, and septic shock had higher VS-I levels than healthy controls ($p < 0.0001$). The levels were also higher in severe sepsis and septic shock patients compared with sepsis patients ($p < 0.0005$) (Table 1 and Figure 2). Although CgA was also increased in patients with severe sepsis and sepsis shock ($p < 0.0001$ vs. controls), there was no significant difference for patients with sepsis (Table 1 and Figure 2). The values of lactates were higher in patients with severe sepsis or sepsis chock than in patients with sepsis (Table 1). There was no significant difference among the three sepsis groups in CRP and leucocyte count (Table 1).

We analyzed the performance of VS-I and CgA to identify patients with severe sepsis and sepsis shock from healthy controls using ROC analysis. As shown on Figure 3A, VS-I had a high discriminative value with an area under ROC (area under curve [AUC]) of 0.96 (95% CI: 0.92–1.00). With a cutoff value of 2.9 ng/mL, the sensitivity was 90% and the specificity 83%. The AUC of VS-I was significantly larger ($p = 0.0006$) than that of CgA (0.77 [0.66–0.89]). We also investigated the performance of VS-I, CgA, lactates, and CRP to differentiate patients with severe sepsis and sepsis shock from patients with sepsis only. As shown on Figure 3B, the AUC was significantly larger ($p = 0.008$) for VS-I [0.83, 95% CI: 0.72–0.94] than for CgA (0.62 [0.47–0.77]). The AUC of VS-I was also greater than that of lactates (0.70 [0.56–0.83]) and CRP (0.70 [0.49–0.90]). The difference of AUC was borderline significant between VS-I and lactates ($p = 0.09$). When the specificity was set at 90%, the sensitivity of VS-I to identify patients with severe sepsis was substantially higher (80%) than that of lactates (35%) or CRP (32%).

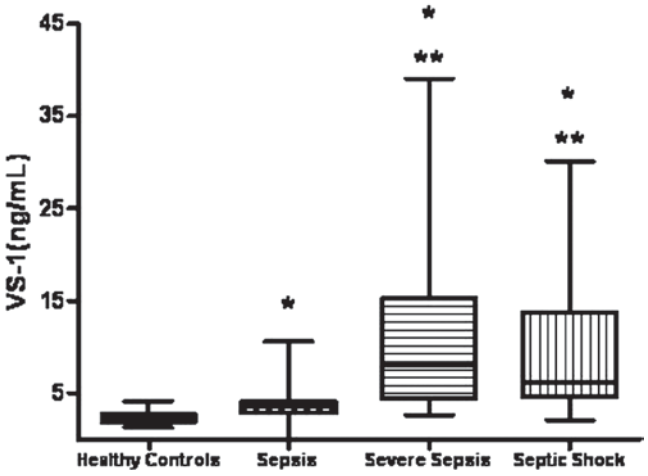

Figure 2. Box and whisker plot of plasma vasostatin-I in 30 healthy controls, 20 patients with sepsis, 20 patients with severe sepsis, and 20 patients with septic shock. *$p < 0.0001$ versus healthy controls, **$p < 0.0005$ versus sepsis group.

Discussion

We developed the first ELISA for VS-I. The results show that the analytical precision and linearity of this assay allow reproducible measurement of plasma VS-I. The assay sensitivity enables the precise measurement of VS-I at levels of 0.34 ng/mL and above. The recovery of spiked recombinant VS-I into plasma demonstrated that the assay recognizes similarly recombinant human VS-I used as a standard and endogenous VS-I. VS-I ELISA does not recognize full-length recombinant CgA nor CgA1-78, which is the first form released from initial cleavage of CgA by PC1/2 (Glattard et al., 2006) and which is converted to the more stable VS-I after cleavage of the 2 lysines at the C-terminal end.

Because VS-I comes from CgA processing, it was important to evaluate the correlation between CgA and VS-I. The results showed that in patients with sepsis a

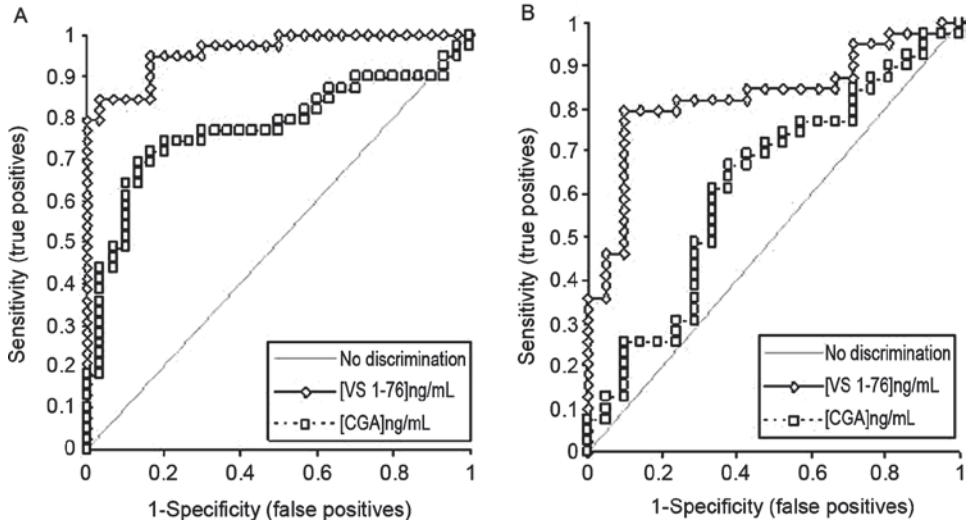

Figure 3. Accuracy of diagnosis of sepsis and its severity by VS-I and CGA. Receiver operating curves (ROC) of vasostatin-I (VS-I) and chromogranin A (CgA) to differentiate patients with severe sepsis and sepsis shock from healthy controls (Panel A) and from patients with sepsis only (Panel B). The area under ROC was significantly larger for VS-I than for CgA (0.96 vs. 0.77, $p = 0.0006$ in Panel A and 0.83 vs. 0.62, $p = 0.008$ in Panel B).

significant but modest correlation between these two markers. It has been shown that CgA is processed by several enzymes within the chromaffin granules both in the C and N-terminal region (Metz-Boutigue et al., 1993) generating several fragments including VS-I. In addition CgA can also be processed in the extracellular space (Metz-Boutigue et al., 1993). CgA processing is thus very complex and likely to vary according the clinical status of the patients. In such a heterogeneous population of patients with sepsis, it is thus not unexpected to observe only a modest association between CgA and VS-I. Conversely, in a more homogeneous population of healthy adults the association between these two biological markers was higher. These data suggest that in patients with sepsis the measurements of VS-I could bring novel and additional information on neuroendocrine metabolism compared with CgA. We observed no significant association of VS-I with serum CRP, lactates, or leucocyte count, which are conventional biological tests used to assess disease activity in sepsis. This indicates that in patients with sepsis, VS-I reflects biological pathways – yet to be elucidated – which are different from systemic inflammation.

We found that VS-I was markedly increased in patients with severe sepsis and septic shock patients compared with healthy controls. The accuracy of VS-I to diagnose severe sepsis as evaluated by ROC analysis was high and substantially greater than that of CgA. The diagnostic accuracy to identify patients with severe sepsis compared with sepsis alone was also higher for VS-I than for CgA, lactates, and CRP. All these findings suggest that VS-I could be more sensitive and specific than the current biological tests to diagnose sepsis and assess its severity.

Our study has strengths and some limitations. This is the first study that describes the development of a new biochemical marker, VS-I, and that assesses its clinical utility in a well-characterized population of patients with sepsis. It also has limitations. The limited number of participants per group requires larger studies to confirm our findings. The lack of longitudinal measurements did not permit the analysis of the time course of VS-I in relation to sepsis diagnosis and following treatment. Finally, because of limited sample availability, we could not measure other potential biomarkers of sepsis severity such as IL-6.

In conclusion, the new ELISA for plasma VS-I is highly specific, reproducible, and sensitive. The clinical data suggest that the measurement of plasma VS-I could bring valuable information on the severity in patients with sepsis.

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Declaration of interest

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References

Becker KL, Snider R, Nylen ES. (2010). Procalcitonin in sepsis and systemic inflammation: a harmful biomarker and a therapeutic target. *Br J Pharmacol* 159:253–264.

Belloni D, Scabini S, Foglieni C, Veschini L, Giazzon A, Colombo B, Fulgenzi A, Helle KB, Ferrero ME, Corti A, Ferrero E. (2007). The vasostatin-I fragment of chromogranin A inhibits VEGF-induced endothelial cell proliferation and migration. *FASEB J* 21:3052–3062.

Bone RC, Sibbald WJ, Sprung CL. (1992). The ACCP-SCCM consensus conference on sepsis and organ failure. *Chest* 101:1481–1483.

Ceconi C, Ferrari R, Bachetti T, Opasich C, Volterrani M, Colombo B, Parrinello G, Corti A. (2002). Chromogranin A in heart failure; a novel neurohumoral factor and a predictor for mortality. *Eur Heart J* 23:967–974.

Conlon JM. (2010). Granin-derived peptides as diagnostic and prognostic markers for endocrine tumors. *Regul Pept* 165:5–11.

Corti A, Sanchez LP, Gasparri A, Curnis F, Longhi R, Brandazza A, Siccardi AG, Sidoli A. (1997). Production and structure characterisation of recombinant chromogranin A N-terminal fragments (vasostatins) – evidence of dimer-monomer equilibria. *Eur J Biochem* 248:692–699.

Glattard E, Angelone T, Strub JM, Corti A, Aunis D, Tota B, Metz-Boutigue MH, Goumon Y. (2006). Characterization of natural vasostatin-containing peptides in rat heart. *FEBS J* 273:3311–3321.

Helle KB. (2010). The chromogranin A-derived peptides vasostatin-I and catestatin as regulatory peptides for cardiovascular functions. *Cardiovasc Res* 85:9–16.

Helle KB, Corti A, Metz-Boutigue MH, Tota B. (2007). The endocrine role for chromogranin A: a prohormone for peptides with regulatory properties. *Cell Mol Life Sci* 64:2863–2886.

Ivády B, Béres BJ, Szabó D. (2011). Recent advances in sepsis research: novel biomarkers and therapeutic targets. *Curr Med Chem* 18:3211–3225.

Lever A, Mackenzie I. (2007). Sepsis: definition, epidemiology, and diagnosis. *BMJ* 335:879–883.

Lugardon K, Chasserot-Golaz S, Kieffer AE, Maget-Dana R, Nullans G, Kieffer B, Aunis D, Metz-Boutigue MH. (2001). Structural and biological characterization of chromofungin, the antifungal chromogranin A-(47-66)-derived peptide. *J Biol Chem* 276: 35875–35882.

Lugardon K, Raffner R, Goumon Y, Corti A, Delmas A, Bulet P, Aunis D, Metz-Boutigue MH. (2000). Antibacterial and antifungal activities of vasostatin-1, the N-terminal fragment of chromogranin A. *J Biol Chem* 275:10745–10753.

Metz-Boutigue MH, Garcia-Sablone P, Hogue-Angeletti R, Aunis D. (1993). Intracellular and extracellular processing of chromogranin A. Determination of cleavage sites. *Eur J Biochem* 217:247–257.

Pierrakos C, Vincent JL. (2010). Sepsis biomarkers: a review. *Crit Care* 14:R15.

Pieroni M, Corti A, Tota B, Curnis F, Angelone T, Colombo B, Cerra MC, Bellocci F, Crea F, Maseri A. (2007). Myocardial production of chromogranin A in human heart: a new regulatory peptide of cardiac function. *Eur Heart J* 28:1117–1127.

Roatta S, Passatore M, Novello M, Colombo B, Dondossola E, Mohammed M, Losano G, Corti A, Helle KB. (2011). The chromogranin A- derived N-terminal peptide vasostatin-I: *In vivo* effects on cardiovascular variables in the rabbit. *Regul Pept* 168:10–20.

Zhang D, Lavaux T, Voegeli AC, Lavigne T, Castelain V, Meyer N, Sapin R, Aunis D, Metz-Boutigue MH, Schneider F. (2008). Prognostic value of chromogranin A at admission in critically ill patients: a cohort study in a medical intensive care unit. *Clin Chem* 54:1497–1503.