Immunofluorometric assay of pepsinogen C and preliminary clinical applications

Eleftherios P. Diamandis, Sheila Nadkarni, Banani Bhaumik, Aly Abdelrahman, Dimitrios N. Melegos, Gudrun Borchert, Margot H. Black, Marta Alonso, Ana Salas, Juan R. de los Toyos, Andres Sampedro, and Carlos López-Otín

We developed mouse monoclonal antibodies (Abs) against pepsinogen C with highly purified antigen isolated from gastric mucosa. The Abs were used to construct a two-site sandwich-type assay for pepsinogen C with time-resolved fluorometry as a detection technique. The assay has a detection limit of 0.1 µg/L and is precise (within-run and day-to-day CVs <11%). We used this assay to measure pepsinogen C in seminal plasma, breast cyst fluid, amniotic fluid, male and female serum, serum from patients with prostate cancer, urine, breast tumor cytosolic extracts, breast milk, and cerebrospinal fluid. Highest pepsinogen C concentrations were in seminal plasma, followed by breast cyst fluid and amniotic fluid. We found no correlation between prostate-specific antigen concentrations and concentrations of pepsinogen C in serum of prostate cancer patients, and concluded that this marker is not useful for either diagnosing or monitoring prostatic carcinoma. The availability of a highly sensitive, reliable, and convenient method for quantifying pepsinogen C will allow investigations into the possible diagnostic value of this analyte in various clinical conditions, including benign breast diseases, breast cancer, fertility, and pregnancy.

INDEXING TERMS: proteases • breast cancer • amniotic fluid • prognostic markers • androgen-regulated genes

1 Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Ave., Toronto, ON M5G 1X5, Canada.
2 Department of Clinical Biochemistry, University of Toronto, 100 College St., Toronto, ON M5G 1L5, Canada.
3 Servicio de Citometria, Universidad de Oviedo 33006, Oviedo, Spain.
4 Departamento de Bioquimica y Biologia Molecular, Facultad de Medicina, Universidad de Oviedo 33006, Oviedo, Spain.
5 Address correspondence to this author at: Depts. of Pathology and Clinical Biochemistry, Mount Sinai Hospital, 600 University Avenue, Toronto, ON M5G 1X5, Canada. Fax 416-588-8628; e-mail epd@eric.on.ca.

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Proteolytic enzymes are thought to be involved in the development of tumor processes because of their role in degradation of the extracellular matrix, thereby facilitating tumor invasion and metastasis [1, 2]. Many studies have shown that a variety of proteolytic enzymes are overproduced either by cancer cells themselves or by the surrounding stromal cells of the host tissue. These proteases include matrix metalloproteinases such as gelatinases, collagenases and stromelysins [3–5], serine proteases such as plasminogen activators [6], cysteine proteases such as cathepsins B and L [7, 8], and aspartic proteases such as cathepsin D [9, 10]. Several clinical studies have shown that the overexpression of these proteinases in breast and other tumors is usually associated with an unfavorable clinical prognosis [11–13].

Pepsinogen C, a member of the aspartic proteinase family of proteolytic enzymes, is the inactive precursor of pepsin C. It is synthesized in the gastric mucosa and secreted into the gastric lumen, where it is converted to the active enzyme under acidic conditions [14–16]. In women with gross cystic disease of the breast, pepsinogen C accumulates in cyst fluid [17]. Moreover, breast carcinomas have the ability to synthesize and secrete pepsinogen C [18]. PCR amplification and Northern blot studies carried out on RNAs obtained from normal and pathological breast tissues have revealed that pepsinogen C is produced by mammary carcinomas and cysts but not by the normal resting mammary gland [18–20]. Pepsinogen C expression by human mammary epithelium may be involved in the development of breast diseases [17–20]. The measurement of pepsinogen C in breast tissue may be of interest as a biochemical marker of the hormonal imbalance underlying these pathologies and that pepsinogen C expression by breast carcinoma cells may be a marker for favorable clinical outcome of this disease [19]. Pepsinogen analysis in serum has also potential for monitoring patients with peptic ulcer and gastric cancer, and in the investigation of Helicobacter pylori infection [21].
Given the potential role of pepsinogen C as a prognostic marker for breast carcinomas and in gastrointestinal disease, it would be beneficial to have a reliable quantitative method for its measurement in biological fluids and tissue extracts. Moreover, the possible presence of pepsinogen C in various biological fluids and tissues has not been examined.

In the current study, we developed a sensitive and specific assay for pepsinogen C by using two monoclonal antibodies (mAbs), time-resolved fluorescence spectrosopy, and Tb chelates as labels.5 Pepsinogen C was measured in various fluids and tissue extracts. This assay is an important tool for elucidating the possible diagnostic or biochemical role of pepsinogen C in breast cyst fluid, seminal plasma, amniotic fluid, and other tissues and fluids.

Materials and Methods

Diflunisal phosphate (DFP) was synthesized in our laboratory (diflunisal, obtained from Sigma Chemical Co., St. Louis, MO). The stock solution of DFP was 0.01 mol/L in 0.1 mol/L NaOH. DFP stock solutions are stable at 4 °C for at least 1 month. Alkaline phosphatase-labeled streptavidin (SA-ALP) was obtained from Jackson ImmunoResearch, West Grove, PA, as a 1 g/L solution. Working SA-ALP solutions were prepared by diluting the stock solution 20 000-fold in a bovine serum albumin (BSA) diluent (described below). White, opaque, 12-well polystyrene microtiter strips were obtained from Dynatech diluent (described below). Several clinical samples were used to examine the presence of pepsinogen C. These included serum samples from male and female hospitalized patients, breast cyst fluids obtained by needle aspiration, breast tumor cytosolic extracts [22, 23], sera from patients with prostate cancer, amniotic fluids, human urines, milks of lactating women, seminal plasmas, cerebrospinal fluids (CSFs), and animal sera. To establish optimal measuring conditions, all samples were tested at various dilutions. Our procedures are in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

INSTRUMENTATION

A time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion International, Kanata, ON, Canada), was used to measure Tb3+ fluorescence in white microtiter wells. The procedure has been described in detail previously [24].

PROCEDURES

Purification of pepsinogen C antigen. Pepsinogen C was purified from human gastric mucosa obtained at autopsy from individuals without gastric disorders [17]. Purity of the obtained zymogen was confirmed by protein sequencing with automatic Edman degradation.

Preparation of pepsinogen C monoclonal and polyonal Abs. For the preparation of a polyclonal Ab against pepsinogen C, we immunized New Zealand rabbits with purified pepsinogen C diluted with complete and incomplete Freund’s adjuvant, with standard procedures [25]. The polyclonal Ab was purified by ion-exchange chromatography. For mAb production, we used Balb/C mice and standard protocols [25]. We selected four clones, reacting with different epitopes, for further study. Relatively large quantities of the four mAbs were prepared by first producing ascites fluid in Balb/C mice and purifying it by protein A affinity chromatography with commercially available reagents (protein A antibody purification kit; Bio-Rad Labs., Richmond, CA).

Coating of microtiter plates with pepsinogen C mAbs. We coated polystyrene microtiter wells by incubating overnight 500 ng/100 μL per well of the coating Ab diluted in a 50 mmol/L Tris buffer, pH 7.80. The wells were then washed six times with the wash solution and blocked for 1 h with 200 μL/well of the blocking solution (10 g/L BSA in 50 mmol/L Tris, pH 7.80). After another six washes, the wells were ready to use.

Biotinylation of pepsinogen C mAbs. Biotinylation of the mAbs was performed with sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-Biotin) obtained from Pierce Chemical, Rockford, IL [26]. In general, we used ~300 μg of NHS-LC-Biotin dissolved in 20–30 μL of dimethyl sulfoxide per milligram of antibody. Before biotinylation, the Ab, dissolved in a 0.1 mol/L phosphate buffer, pH 7.4, at a concentration of ~1 g/L, was diluted with an equal volume of 0.5 mol/L carbonate buffer solution, pH 9.5. After biotinylation for 1 h at room temperature, the Ab was stored at 4 °C and used without any further purification. The concentration of the stock biotinylated Ab solution was ~0.5 g/L.

CLINICAL SAMPLES

Several clinical samples were used to examine the presence of pepsinogen C. These included serum samples from male and female hospitalized patients, breast cyst fluids obtained by needle aspiration, breast tumor cytosolic extracts [22, 23], sera from patients with prostate cancer, amniotic fluids, human urines, milks of lactating women, seminal plasmas, cerebrospinal fluids (CSFs), and animal sera. To establish optimal measuring conditions, all samples were tested at various dilutions. Our procedures are in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

5 Nonstandard abbreviations: mAb, monoclonal antibody; DFP, diflunisal phosphate; SA-ALP, streptavidin conjugated to alkaline phosphatase; BSA, bovine serum albumin; CSF, cerebrospinal fluid; NHS-LC-Biotin, sulfosuccinimidyl 6-(biotinamido) hexanoate; and PSA, prostate-specific antigen.
Pepsinogen C calibrators. Pepsinogen C calibrators of 0, 0.5, 2, 10, 50, and 200 μg/L were prepared by diluting highly purified pepsinogen C in a 50 mmol/L Tris buffer, pH 7.80, containing 60 g of BSA and 0.5 g of sodium azide per liter.

Pepsinogen C assay. Calibrators or samples (100 μL) were pipetted into coated microtiter wells and 50 μL of the biotinylated Ab solution diluted 1000-fold in assay buffer was added (~25 ng of biotinylated Ab per well). The plates were incubated with mechanical shaking for 1 h at room temperature and then washed six times. To each well we then added 100 μL of SA-ALP conjugate diluted 20 000-fold in the SA-ALP diluent, incubated for 15 min as described above, and then washed six times. To each well we then added 100 μL of the 1 mmol/L DFP working substrate solution and incubated for 10 min as described above. We added 100 μL of developing solution to each well, mixed by mechanical shaking for 1 min, and measured the fluorescence with the time-resolved fluorometer [24]. The calibration curve and data reduction were performed automatically by the CyberFluor 615 Immunoanalyzer.

Prostate-specific antigen (PSA) assay. PSA was measured with an immunoassay technique described in detail elsewhere [27].

Results

AB SELECTION AND ASSAY OPTIMIZATION

Four mouse mAbs and one rabbit polyclonal Ab were prepared and used to develop a pepsinogen C immunofluorometric assay. Each mAb was used for pepsinogen C capture, after immobilization to microtiter wells. Also, each mAb was biotinylated and tested as a detection Ab, in combination with ALP-conjugated streptavidin, in accordance with general procedures and principles [26, 28]. The polyclonal Ab was used for detection in combination with ALP-conjugated goat anti-rabbit immunoglobulin. The detection of ALP activity was accomplished with time-resolved fluorometry [24]. Among all tested combinations, best results were obtained with the mAb 1.3C3.91 as coating Ab and the mAb 1.9H12.78 as biotinylated detection Ab. A one-step assay format was as sensitive and faster than a two-step assay format. Other variables, including sample volume, incubation times, amount of reactants, and composition of buffers, were optimized with a previously published optimization strategy [28]. The final conditions are described in Materials and Methods.

CALIBRATION CURVE, DETECTION LIMIT, PRECISION, HOOK EFFECT

A typical calibration curve of the proposed assay is shown in Fig. 1. The detection limit, defined as the concentration of pepsinogen C corresponding to the fluorescence of the zero calibrators plus two SDs, is 0.1 μg/L. Within-run and between-run precision was assessed at various pepsinogen C concentrations between 0.5 and 200 μg/L and with various samples containing pepsinogen C, as shown in Table 1. In all cases, CVs were between 3% and 11%.

| Table 1. Analysis of pepsinogen C in various fluids. |
|-----------------------------------------------|-------------------|-------------------|-------------------|-------------------|
| Sample                                      | Range            | Mean (SD)          | Median           | n       |
| Seminal plasma                              | 26 400–148 000   | 67 700 (37 400)    | 52 919           | 15      |
| Breast cyst fluid                           | 0.6–13 000       | 277 (2830)         | 2056             | 53      |
| Amniotic fluid                              | 1.4–41           | 12.1 (11.4)        | 6.7              | 29      |
| Male serum                                  | 0–14             | 2.0 (2.8)          | 0.90             | 42      |
| Male serum, prostate cancer                 | 0–38             | 2.2 (6.2)          | 0.60             | 45      |
| Female serum                                | 0–5              | 1.3 (1.3)          | 0.85             | 42      |
| Urine                                       | 0–18             | 2.0 (4.0)          | 1.8              | 12      |
| Breast tumor cytosolic extracts             | 0.2–8.6          | 2.1 (1.8)          | 1.7              | 42      |
| Breast milk                                 | 0–0.6            | <0.1               | <0.1             | 5       |
| CSF                                         | 0–0.5            | <0.1               | <0.1             | 6       |

a No. of samples tested.
consistent with the precision of typical microtiter plate-based immunoassays. The hook effect was checked up to a pepsinogen C concentration of 50,000 μg/L; all concentrations tested read >200 μg/L, suggesting no hook effect up to this concentration.

SPECIFICITY

Specificity was assessed with various tests. (a) We analyzed undiluted animal sera from mice, rabbits, goats, horses, rats, and calves, and none of them gave measurable pepsinogen C concentrations. (b) We checked for cross-reactivity with PSA (another major constituent of seminal plasma) and found no detectable cross-reactivity up to 50,000 μg/L (the highest concentration tested). (c) We identified female sera, male sera, amniotic fluids, seminal plasmas, and breast cyst fluids with relatively high concentrations of pepsinogen C and fractionated them with HPLC on a gel filtration column [29]. All fractions were then analyzed for pepsinogen C. In all fluids, we obtained a single immunoreactive peak with a molecular mass of 35–40 kDa, consistent with the molecular mass of pepsinogen C (Fig. 2). One of 10 breast cyst fluids fractionated contained a minor immunoreactive peak with a molecular mass of 100–150 kDa (Fig. 2). The nature of this peak is unknown (see also below).

DILUTION EXPERIMENTS

We analyzed samples with relatively high pepsinogen C concentrations (amniotic fluid, breast cytosol, breast cyst fluid, and seminal plasma) at various dilutions and calculated the percent of expected value. The diluent was the same as the calibrator diluent. For amniotic fluid, the percent of expected value at dilutions of 32, 16, 8, 4, 2, and undiluted were 100% (by definition this was considered the 100% of expected value), 100%, 100%, 100%, 83%, and 43%, respectively. For breast cytosolic extracts, the percent of expected value was 99–100% for all dilutions (undiluted to 32-fold). For the breast cyst fluid, the percent of expected value was 99–100% for all dilutions (undiluted to 32-fold). For the breast cyst fluid, the percent of expected value was 99–100% for all dilutions (undiluted to 32-fold). The percent of expected value was 100% at any dilution >1000-fold. Lower dilutions could not be used because this fluid has pepsinogen C concentrations much higher than our highest calibrator. Efforts to even further improve the dilution linearity of the assay by incorporating sodium dodecyl sulfate in the assay buffer, at concentrations 0.02–0.2%, were unsuccessful (data not shown).

RECOVERY

Recovery was checked by supplementing purified pepsinogen C at two concentrations (10 or 20 μg/L) in various matrices and analyzing them with the proposed method. Matrices supplemented included a 60 g/L BSA solution, goat, horse, mouse, and rabbit serum, two male and two female human sera, two amniotic fluids, two CSFs, and two human urines. The 60 g/L BSA solution gave 100% recovery. None of the other matrices gave complete recovery. The percent recovery range was 10–57% for the animal sera, 30–50% for the human sera, 70–90% for the amniotic fluids, 60–80% for the CSFs, and 40–50% for the urines. To check if the recovery in serum was from matrix effects in the samples, we modified the assay to use less serum (50 μL, 25 μL, or 10 μL instead of 100 μL), and more assay buffer (100 μL instead of 50 μL) and incubation time (2 h instead of 1 h). None of these manipulations increased recovery to 100%. We furthermore checked if the low recovery was due to a time-dependent proteolytic digestion of pepsinogen C, by analyzing the supplemented samples either immediately after supplementation or after incubation for 5 h at 37 °C. Recovery was the same with or without the incubation.

To check if pepsinogen C interacts with components of the biological fluids leading to its inactivation or complexation, we prepared pepsinogen C radioactively labeled with 125I by the chloramine-T method. This preparation was further purified by HPLC to isolate pepsinogen C monomer with a molecular mass of ~35
The monomer was then added to a BSA solution (control), three human sera, one amniotic fluid, and one breast cyst fluid. After incubation at room temperature for 2–18 h, these samples were separated by gel filtration HPLC and the radioactivity of fractions were counted. Partial results are shown in Figs. 3 and 4.

A BSA matrix contains two peaks of radioactivity, one for pepsinogen C (around fraction 38, molecular mass ~35 kDa) and one for free $^{125}$I (around fraction 49, molecular mass <1000 Da). In all three human sera, we detected a new peak of radioactivity (around fraction 22, molecular mass ~300–600 kDa). The same peak, but at a lower concentration, was also seen in the amniotic fluid and the breast cyst fluid. Additionally, a new peak (around fraction 30, molecular mass 100–150 kDa) was seen only in the breast cyst fluid sample (Fig. 4). Interestingly, in the supplemented sera, in comparison with the BSA control supplement, the peak radioactivity of pepsinogen C monomer decreased by ~50% and the peak radioactivity of free $^{125}$I increased by ~50% (Fig. 3). The HPLC data with radioactive pepsinogen C are consistent with (a) binding of pepsinogen C to serum, amniotic fluid, and breast cyst fluid components, and (b) possible proteo-

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Fig. 3. Radioactivity of all fractions of a 60 g/L BSA solution ($\square$) or a male serum ($\Delta$) supplemented with $^{125}$I-labeled pepsinogen C, incubated for 2–18 h, and separated with gel filtration HPLC.

In the case of BSA solution, there is a peak at fraction 38 (peak 2) representing free pepsinogen C with molecular mass of 35–40 kDa and another peak around fraction 49 (peak 3) representing free $^{125}$I. In male serum, there is an additional peak around fraction 22 (peak 1) corresponding to a molecular mass of 300–600 kDa. In panel B, the same chromatographic separation was drawn with an expanded y-scale to indicate the appearance of peak 1 and the increase of peak 3. For more discussion, see text.

Fig. 4. Radioactivity of all fractions of a 60 g/L BSA solution ($\square$) or breast cyst fluid ($\cdot$) supplemented with $^{125}$I-labeled pepsinogen C, incubated for 2–18 h, and separated with gel filtration HPLC.

In the case of BSA solution, there is a peak at fraction 38 (peak 2) representing free pepsinogen C with molecular mass of 35–40 kDa and another peak around fraction 49 (peak 3) representing free $^{125}$I. In the breast cyst fluid, there are two additional peaks around fraction 22 (peak 1) corresponding to a molecular mass of 300–600 kDa and around fractions 30–31 (peak 4) corresponding to a molecular mass of 100–150 kDa. In panel B, the same chromatographic separation was drawn with an expanded y-scale to indicate the appearance of peaks 1 and 4 and the increase of peak 3. For more discussion, see text.
lytic degradation of pepsinogen C after supplementing, with release of small peptides containing $^{125}$I and eluting at around fraction 49.

**PEPSINOGEN C IN BIOLOGICAL FLUIDS AND EXTRACTS**

To obtain preliminary information on the presence of pepsinogen C in biological fluids, we analyzed various clinical samples as shown in Table 1. The highest concentration of pepsinogen C was found in seminal plasma (150 000 μg/L). The second highest concentration was seen in breast cyst fluid. Much lower but still measurable concentrations in all samples were seen in amniotic fluids. In male and female serum, the concentrations were relatively very low. We found a trend for higher pepsinogen C concentrations in serum of males with age [pepsinogen C (μg/L) = −1.4 ± 0.06; r = 0.35, P = 0.03] but not in females (r = 0.18, P = 0.12). Among 44 patients with prostate cancer and serum PSA between 50 and 4000 μg/L, we found no correlation between serum pepsinogen C and PSA (r = 0.03, P = 0.85) and no indication that pepsinogen C is increased in the serum of these patients in comparison with control males (Table 1).

**Discussion**

The gene for pepsinogen C has been cloned and sequenced [30]. Pepsinogen C is the inactive precursor of pepsin C, a member of the aspartic proteinase family of proteolytic enzymes. This molecule is synthesized primarily by the chief cells of the gastric glands and is secreted into the gastric lumen where it is converted to the active form under acidic conditions. The cDNA of pepsinogen C predicts a 388-residue amino acid sequence consisting of a signal sequence of 16 amino acids, an activation peptide of 43 residues, and the mature pepsin of 329 residues.

Pepsinogen C is reportedly present at high concentrations in breast secretions and in breast tumor extracts. Clinical studies have indicated that pepsinogen C is a favorable prognostic indicator in breast cancer [19]. Studies on the regulation of the pepsinogen C gene have revealed that this molecule is upregulated by androgens, glucocorticoids, and progestins [20]. Interestingly, pepsinogen C is not the only molecule that has been isolated from gastric mucosa and breast tissue. Another protein, pS2, has also been found in these two tissues and is a favorable prognostic indicator in breast cancer, but its regulation is mediated through estrogens [31].

Recently, it became apparent that a group of molecules that are present at high concentrations in seminal plasma can also be found in breast secretions, breast tissue extracts, and breast cancer cell lines. Among these molecules are pepsinogen C, PSA, apolipoprotein D, and Zn-α2-glycoprotein. It now appears likely that many seminal plasma constituents are also present in breast secretions and extracts as well as in amniotic fluid [32]. We reported presence of prostaglandin D synthase in seminal plasma, amniotic fluid, and breast tumor extracts [29].

Until now, pepsinogen C was usually studied at the mRNA level with molecular techniques such as PCR or immunohistochemistry with polyclonal Abs. To facilitate more studies into the production, regulation, and possible clinical significance of pepsinogen C measurements in various fluids and extracts, we developed a highly sensitive and specific immunofluorometric method with mAbs. These Abs were raised by immunizing mice with highly purified preparations of pepsinogen C isolated from gastric mucosa. Among the mAbs developed, we selected two that were suitable for a sandwich-type assay of pepsinogen C. We presented data indicating that the newly developed assay is highly sensitive and specific for pepsinogen C.

The recovery of added pepsinogen C was <100% for all biological samples tested. Our experiments show that the matrix effect may not be the problem because use of less sample volume or more assay buffer did not improve results. Our data with radioactive pepsinogen C suggest that this molecule binds to serum components and forms high-molecular-mass complexes. One complex with molecular mass ≥300 000 may represent pepsinogen C bound to proteinase inhibitors, as is the case with PSA [27]. In breast cyst fluid, pepsinogen C binds to an unknown component, forming a 100–150 000-Da complex recognizable by our assay (Figs. 2 and 4). We also have indication for pepsinogen C proteolysis with release of low-molecular-mass radioactive fragments.

On the basis of analysis of a large number of biological fluids, the highest concentrations of pepsinogen C were found in seminal plasma. However, the concentrations of this molecule are ~10 times lower than the concentrations of PSA in this fluid. We are now in the process of establishing the value of measuring pepsinogen C in seminal plasma as an aid in the differential diagnosis of male infertility. We also speculated that pepsinogen C concentrations in serum may be increased in patients with prostate cancer or benign prostatic hyperplasia, situations that would be similar to the measurements of PSA. Pepsinogen C has been reported to be produced by the prostate gland [16]. However, we did not observe any significant increases of serum pepsinogen C in patients with prostate cancer (Table 1). Moreover, we found no correlation between serum pepsinogen C concentrations and serum PSA concentrations in patients with prostate cancer and very high PSA values. We thus conclude that the measurement of pepsinogen C for either diagnosing or monitoring prostate cancer is not useful. Similar conclusions were reported for prostaglandin D synthase, which is another constituent of seminal plasma and appears to be produced not by the prostate gland but by the seminal vesicles [29].

We found relatively high concentrations of pepsinogen C (up to 13 000 μg/L) in breast cyst fluid. Quantitative analysis of pepsinogen C in this fluid is currently being performed to investigate the possible diagnostic value of this marker in benign breast diseases. We found no major differences between male or female serum pepsinogen C...
concentrations. Clinical studies assessing the possible prognostic value of quantitatively measured pepsinogen C in tumor extracts from patients with breast cancer are now underway. It has been previously shown that pepsinogen C assessment by immunohistochemistry has prognostic value. Higher concentrations of this marker are associated with improved disease-free and overall survival [19].

We here report for the first time that pepsinogen C is present at easily measurable concentrations in all amniotic fluids tested. We are currently investigating the value of this marker in diagnosing fetal abnormalities by measuring pepsinogen C either in maternal serum or amniotic fluid.

In summary, we present a quantitative immunological assay for measuring pepsinogen C concentrations in various biological fluids. We anticipate that the availability of this highly sensitive and specific method will further facilitate investigations into the biology of pepsinogen C and the possible diagnostic value of measuring pepsinogen C in biological fluids including seminal plasma, breast cyst fluid, amniotic fluid, and breast tumor extracts.

References