Assay for Measurement of Intact B-Type Natriuretic Peptide Prohormone in Blood

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Background: B-Type natriuretic peptide (BNP_1–32) as well as the N-terminal fragment of the prohormone containing residues 1–76 (NT-proBNP_1–76), both cleavage products of the precursor proBNP_1–108, are reported to be powerful markers for prognosis and risk stratification of heart failure. However, the intact precursor also circulates in the bloodstream. Assays for the detection of these cleavage products have been developed, but most of these assays may overestimate the concentrations of the cleavage products because they also measure the precursor form. It is therefore important to develop an immunoassay that specifically measures solely proBNP_1–108 in plasma.

Methods: After carefully designing the peptide used to immunize mice, we selected a specific monoclonal antibody (mAb Hinge76) that recognizes the cleavage site of proBNP_1–108, an epitope present only in the precursor form. mAb Hinge76 recognizes recombinant proBNP_1–108 in a dose-dependent manner, without any significant cross-reactivity with either recombinant NT-proBNP_1–76 or synthetic BNP_1–32. By combining mAb Hinge76 with a polyclonal antibody directed against BNP_1–32, we were able to set up a proBNP_1–108-specific sandwich immunoassay able to confirm the presence of proBNP_1–108 in blood samples.

Results: From a cohort of 50 healthy persons and 170 patients with congestive heart failure (CHF), our assay was able to differentiate healthy individuals from CHF patients (P < 0.005). Interestingly, plasma proBNP_1–108 concentrations were correlated with New York Heart Association classification. Moreover, a close relationship between proBNP_1–108 and BNP_1–32 concentrations may exist, as a good correlation (r^2 = 0.89) was obtained when their respective concentrations were compared.

Conclusion: mAb Hinge76 is the first proBNP_1–108-specific mAb produced that allows accurate estimation of proBNP_1–108 concentrations in plasma.

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In 1981, de Bold et al. (1) provided the definitive demonstration of the endocrine function of the heart. This was followed by the identification of the “brain natriuretic peptide”, or B-type natriuretic peptide (BNP), in 1988 (2, 3). This natriuretic peptide was originally discovered in the porcine brain, but its name is a misnomer as the protein is synthesized, stored, and released mainly in the ventricular myocardium (4). This hormone promotes natriuresis and diuresis, acts as a vasodilator, and antagonizes the vasoconstrictor effects of the renin-angiotensin-aldosterone system (5).

The human gene for BNP codes a 134–amino acid preproBNP precursor, which after removal of a 26-amino acid signal peptide gives rise to a 108-amino acid proBNP polypeptide (proBNP_1–108) (6). Further processing releases a mature 32-amino acid BNP molecule (BNP_1–32), which corresponds to the C-terminal sequence of the human proBNP and a 76–amino acid N-terminal fragment (NT-proBNP_1–76). The former exhibits the biological activity, whereas no defined biological function has been found to be associated with the latter. The processing site
in proBNP<sub>1–108</sub> occurs immediately downstream from the Arg<sub>73</sub>-X-X-Arg<sub>76</sub> sequence, at a cleavage site similar to that recognized by the ubiquitous endoprotease furin (7, 8). However, other endoproteases, such as corin (9, 10), or prohormone convertases (11) may be involved in the posttranslational maturation of proBNP<sub>1–108</sub>. Indeed, the cardiac processing of proBNP<sub>1–108</sub> is still poorly characterized, as is that of the proBNP-derived peptide molecular forms circulating in the bloodstream. It is now well established that BNP<sub>1–32</sub> is one of the major circulating forms (12, 13), and it seems likely that BNP<sub>1–32</sub> may undergo deletion of the 2 N-terminal amino acid residues (Ser-Pro) (14). In addition to BNP<sub>1–32</sub> other forms of proBNP-derived peptides have been reported to circulate in the bloodstream, including the intact precursor (13) and N-terminal proBNP fragments (15, 16). Using gel-filtration HPLC followed by immunoassays for BNP<sub>1–32</sub> and proBNP<sub>1–108</sub>, Shimizu et al. (13) analyzed plasma samples from heart failure patients and reported the existence of 2 molecular forms, high–molecular-mass BNP, corresponding to the precursor proBNP<sub>1–108</sub>, and low–molecular-mass BNP, corresponding to BNP<sub>1–32</sub>.

Over the past decade, it has been demonstrated that the B-type natriuretic peptides have great pathophysiologic importance and therefore are of great significance in the diagnosis of heart failure as well as in risk stratification (17, 18). Growing interest in the clinical determination of B-type natriuretic peptides has led to the development of fully automated immunoassays for BNP<sub>1–32</sub> and for NT-proBNP<sub>1–76</sub>. Studies have compared the clinical performance characteristics of both BNP<sub>1–32</sub> and NT-proBNP<sub>1–76</sub> assays (19–21). Overall, their performances are comparable, and both have become increasingly integrated into routine clinical practice. Nevertheless, the BNP<sub>1–32</sub> and NT-proBNP<sub>1–76</sub> assay characteristics need to be better understood for their optimal use in a clinical setting. This is why the IFCC Committee on Standardization of Markers of Cardiac Damage has prepared guidelines for analytical validation of BNP<sub>1–32</sub> and NT-proBNP<sub>1–76</sub> assays (22). One of these recommendations is to analyze the capacity of the BNP<sub>1–32</sub> and the NT-proBNP<sub>1–76</sub> assays to cross-react with proBNP<sub>1–108</sub> and its multimers, a possible source of result disagreement. Indeed, because of the different sets of antibodies used in these different immunoassays, discrepancies between the reported concentrations of proBNP-derived peptides in healthy persons as well as in heart failure patients have been reported (23).

In this situation, the availability of an alternative assay that would specifically quantify the precursor form in biological samples is desirable.

The aims of the present study were to obtain a monoclonal antibody (mAb) specific for proBNP<sub>1–108</sub> without cross-reactivity with either BNP<sub>1–32</sub> or NT-proBNP<sub>1–76</sub> and to use this specific mAb in an assay for proBNP<sub>1–108</sub> measurement in plasma samples.

### Materials and Methods

#### Blood Samples

For this study, we used 250 venous blood samples from patients who had been admitted to the Lapeyronie Hospital for congestive heart failure (CHF). For 79 patients, only the BNP<sub>1–32</sub> value was known, whereas for the other 171, the following characteristics were recorded: age, sex, clinical severity characterized according to the New York Heart Association (NYHA) functional class ranking (24), and the left ventricular ejection fraction (LVEF) evaluated with echocardiography, scintigraphy, or angiography. These 171 patients were split into NYHA class I (n = 17; median age, 61 years (range, 37–96 years); 65% males; median LVEF, 58% (range, 50%–65%)), NYHA class II (n = 69; median age, 64 years (range 21–83 years); 81% males; median LVEF, 42% (range, 13%–66%); 50% ischemic and 9% valvular troubles), NYHA class III (n = 67; median age, 71 years (range, 37–92 years); 76% males; median LVEF, 38.5% (range, 15%–78%); 48% ischemic and 18% valvular troubles), and NYHA class IV (n = 18; median age, 72 years (range, 54–91 years); 83% males; median LVEF, 34.8% (range, 18%–50%); 44% ischemic and 39% valvular troubles). Blood samples were collected in EDTA-containing plastic Vacutainer Tubes (Becton Dickinson). Plasma BNP<sub>1–32</sub> concentrations were measured by a 2-site sandwich chemiluminescent immunoassay on the Beckman Coulter-Access platform (Biosoite Incorporated) within 2 h after blood collection. According to the manufacturer, the limit of detection is 1 ng/L with 95% confidence. Plasma samples were then frozen without protease inhibitors and stored at −80 °C. As control samples, we obtained from Scipac Ltd. (United Kingdom) 50 EDTA-plasma samples from healthy blood donors, without clinical evidence of cardiac disease or a history of chronic illness. The age range of the donors (50–80 years; median of ages, 60 years) matched quite well with that of the heart failure patients, and the sex partitioning was respected (76% males). All studies using human blood samples were approved by the Institutional Review Board of the Arnaud de Villeneuve Hospital. However, because leftover blood from routine collections was used for all samples and no patient identifiers were used, informed consent was deemed unnecessary.

#### Antigens

Human recombinant proBNP<sub>1–108</sub> and NT-proBNP<sub>1–76</sub> proteins were purchased from Hytest. Both were isolated from recombinant Escherichia coli cultures. The resulting proteins had purities of 95% and 98%, respectively, according to Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proBNP<sub>1–108</sub> stock solution was 1 g/L in 50 mmol/L Tris acetate (pH 7), 750 mmol/L NaCl, whereas the NT-proBNP<sub>1–76</sub> stock solution was at 1 g/L in 10 mmol/L potassium phosphate (pH 7.4), 150 mmol/L NaCl. BNP<sub>1–32</sub> was purchased from Sigma and had been synthesized chemically, with a purity >97%, as determined by HPLC. Initially lyophilized, the
BNP$_{1-32}$ was dissolved in phosphate-buffered saline (PBS), pH 7.4, at a final concentration of 1 g/L. Different proBNP-derived peptides containing in their sequence the cleavage site (Arg$^{66}$-Ser$^{77}$) of proBNP$_{1-108}$ with a variable number of flanking residues were synthesized by use of an automated peptide synthesizer (Pioneer). Coupling reactions were performed with the Fmoc/tBu strategy. The peptides were purified on a reversed-phase C$_{18}$ column (Prep-Pak; particle size, 15 μm; pore size, 300 Å; Waters) in a Prep LC 4000 system (Waters) after trifluoroacetic acid cleavage. The purity of the peptides was established by analytical reversed-phase HPLC (DeltaPak C$_{18}$ column; particle size, 15 μm, pore size, 300 Å; Waters). The relative molecular masses of the peptides were determined by electrospray ionization mass spectrometry (LCT; Micromass). For coupling to the keyhole limpet hemocyanin molecule (KLH), the peptides were synthesized with the addition of a Cys residue at their amino terminus. Coupling took place between the Cys-SH group and a primary amino group of the carrier protein and was performed with the heterobifunctional coupling reagent sulfo-SMCC (Pierce), according to the manufacturer’s instructions.

**MONOCLONAL ANTIBODY**

Production of the mAb was carried out by immunization of 5-week-old BALB/c mice (Charles River Laboratories) with 1 subcutaneous injection followed by 2 intraperitoneal injections, at 3-week intervals, of one specific peptide coupled to the KLH. To that end, the KLH-conjugated peptide was emulsified in the same amount of Freund’s complete adjuvant for the first injection and of Freund’s incomplete adjuvant for the 2 subsequent booster injections. The mice were bled on day 78, and serum immunoreactivity was tested by ELISA against recombinant proBNP$_{1-108}$ and NT-proBNP$_{1-76}$ and BNP$_{1-32}$. The mouse that showed the most specific immune response for proBNP$_{1-108}$ was sacrificed, and its splenocytes were isolated and fused according to the procedure described by Kohler and Milstein (25). Hybridomas were screened for antibody production against proBNP$_{1-108}$ as follows. Briefly, plates (Nunc Maxisorp) were coated overnight at 4 °C with recombinant proBNP protein (0.5 mg/L) or an irrelevant protein (GST); Sigma) as control. The plates were blocked by addition of 100 μL of 0.01 mol/L PBS (pH 7.4) containing 1 mL/L Tween 20 (PBS-T); Sigma; fraction V) and incubated for 1 h at 37 °C. The plates were washed 3 times with PBS-T, and 75 μL of each hybridoma culture supernatant plus 25 μL of PBS-T containing 1 g/L BSA were added to each well. The plates were then incubated for 2 h at room temperature and washed 3 times with PBS-T. Peroxidase-conjugated donkey anti-mouse whole-molecule IgG (Jackson) diluted 1:2000 in PBS-T containing 1 g/L BSA was added to each well, and the plates were incubated for 1 h at room temperature. After 3 washings with PBS-T, substrate mixture (100 μL of 0.5 g/L o-phenylenediamine and 0.4 mL/L hydrogen peroxide in 0.1 mol/L citrate buffer, pH 4) was added to each well, and the plates were incubated in the dark at room temperature for 20 min. The reaction was stopped with 50 μL of 2 mol/L H$_2$SO$_4$, and the absorbances were measured at 490 nm with the PR2100 microplate reader (Bio-Rad Laboratories). Positive hybridomas were subcloned by limiting dilution, and those showing the highest reactivity and the best specificity for proBNP$_{1-108}$ were selected for further study. They were cultured in vitro under conditions in which mAbs are naturally secreted into the culture medium and in which high mAb concentrations are expected. The mAbs were further purified on a protein G column (Amersham), and their isotypes were determined by use of the Mouse Monoclonal Antibody Isotyping Kit (Roche).

To simplify the study, we focused our attention on mAb Hinge76, which showed the best affinity and specificity for the intact proBNP$_{1-108}$ protein. This was evaluated by ELISA as follows: Plates were coated overnight at 4 °C with recombinant proBNP$_{1-108}$, BNP$_{1-32}$, and NT-proBNP$_{1-76}$ proteins as well as with an irrelevant protein (GST) at a similar molar concentration (40 nmol/L). The plates were blocked by the addition of 100 μL of PBS-T containing 10 g/L BSA and incubated for 1 h at 37 °C. The plates were washed 3 times with PBS-T, and mAb Hinge76 diluted in PBS-T containing 1 g/L BSA was added to each well. mAb Hinge76 was then detected as described above.

**EPI TOPE MAPPING**

Overlapping pentadecapeptides frameshifted by 3 residues and spanning the entire proBNP$_{1-108}$ sequence were prepared on cellulose membranes (Intavis) by the Spot technique (26) with an ASP222 robot (Abimed) as described previously (27). Once the epitope was identified, series of analog peptides were prepared in which each position of the reactive sequence was substituted by an alanine. The set of membrane-bound peptides was probed by incubation for 90 min at 37 °C with 1 mg/L mAb Hinge76 in Tris-buffered saline containing 1 mL/L Tween 20, 0.1 g/L saccharose, and Blocking Buffer (Roche) diluted 1:50 in Tris-buffered saline–Tween. Binding of mAb Hinge76 to peptides was detected with an alkaline phosphatase–conjugated anti-mouse IgG antibody (Sigma) followed by the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate disodium salt-thiazolyl blue tetrazolium bromide (BCIP/MTT). Membranes were scanned with a CanonScan N650U (Canon), and spot intensity was registered by the Scion Image software. Membranes were regenerated as described previously (27).

**DIG ESTION BY FURIN**

Recombinant proBNP$_{1-108}$ at 100 μg/L was dialyzed against 150 mmol/L NaCl–5 mmol/L CaCl$_2$–50 mmol/L Tris-HCl, pH 7.5. Digestion was performed in the same buffer in the presence of 1 U of recombinant furin (Sigma). An aliquot was removed after 1, 2, and 3 h of digestion at 37 °C. The reaction was stopped by freezing the sample to −80 °C. The samples were then loaded in
duplicate on 2 sodium dodecyl sulfate–polyacrylamide gels (16%), subjected to electrophoresis, transferred to nitrocellulose, and incubated with either the rabbit polyclonal antibody raised against BNP$_{1–32}$ (Strategic Biosolutions; 1 mg/L) or mAb Hinge76 (1 mg/L), followed by incubation with peroxidase-conjugated anti-rabbit immunoglobulin (1:8000 dilution; Jackson ImmunoResearch) or by peroxidase-conjugated anti-mouse immunoglobulin (1:3000 dilution; Jackson ImmunoResearch), respectively. Antibody binding was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**INTACT mAb proBNP IMMUNOASSAY**

mAb Hinge76 was coated at 5 mg/L on microplates. The wells were blocked by the addition of 250 μL of PBS-T containing 10 g/L nonfat dried milk and incubated for 1 h at 37 °C. The plates were washed 3 times with PBS-T, and 50 μL of patient sample diluted in 50 μL of PBS-T containing 1 g/L nonfat dried milk was added to each well. The plates were incubated for 2 h at room temperature under gentle shaking and washed 3 times with PBS-T. Bound proBNP$_{1–108}$ molecule was then revealed by a rabbit polyclonal antibody directed against the BNP part (Strategic Biosolutions), used at 1 mg/L diluted in PBS-T containing 0.1 g/L BSA and incubated for 1 h at room temperature. Peroxidase-conjugated goat anti-rabbit whole-molecule IgG (Sigma) diluted 1:4 000 in PBS-T containing 1 g/L nonfat dried milk as well as irrelevant murine IgG at 150 mg/L was added to each well, and the plates were incubated for 1 h at room temperature. After 3 washings with PBS-T, substrate mixture (100 μL of 0.5 g/L o-phenylenediamine and 0.4 mL/L hydrogen peroxide in 0.1 mol/L citrate buffer, pH 4) was added to each well, and the plates were incubated in the dark at room temperature for 20 min. The reaction was stopped by addition of 50 μL of 2 mol/L H$_2$SO$_4$, and the absorbances at 490 nm were measured. Human recombinant proBNP$_{1–108}$ from HyTest was used as calibrator because it has the advantages of having no His tag at its extremities and having the same sequence as the natural protein. We constructed the calibration curve by diluting the highest calibrator, human recombinant proBNP$_{1–108}$ at a concentration of 20 000 ng/L in 500 mL/L horse plasma, in PBS-T containing 1 g/L nonfat dried milk and then diluting in the same buffer mixture to prepare calibrators with final concentrations of 10 000, 7500, 5000, 2500, 1250, 625, 313, and 156 ng/L. In preliminary experiments, we verified that the horse plasma BNP$_{1–32}$ concentration was <100 ng/L, which is the decision threshold in the Access BNP assay. The within-run (n = 12) and between-day imprecision (n = 10 days) was determined at 3 calibrator concentrations (5000, 1000, and 500 ng/L), prepared by diluting calibrator in 500 mL/L horse plasma in PBS-T containing 1 g/L nonfat dried milk.

**STABILITY**

We tested analyte stability in 4 different plasma samples, obtained from CHF patients classified as NYHA class IV, stored at 4 °C and at room temperature (22 °C). Immediately after blood centrifugation, an aliquot (control) was removed, and other aliquots were left standing for 10 and 24 h at the above temperatures. The different aliquots were stored at −80 °C until determination of proBNP$_{1–108}$ concentrations at each time point, in duplicate.

**STATISTICAL ANALYSIS**

Plasma concentrations of proBNP$_{1–108}$ and BNP$_{1–32}$ did not follow a gaussian distribution. Thus, their concentrations are reported as the median and interquartile range (25th–75th percentiles). An independent Student t-test was carried out to compare proBNP$_{1–108}$ and BNP$_{1–32}$ concentrations within each NYHA class as well as within the different LVEF subgroups. The results were regarded as statistically significant for a P value <0.05. Moreover, to assess the relationship between these 2 analytes, we log-transformed their concentrations before statistical analysis and performed a linear regression analysis.

**Results**

Among the different immunogenic peptides, one peptide, with a sequence of Cys-YTLRAPS PKMVGQ, appeared to be the most appropriate candidate, as most immunized mice produced antibodies specific for the proBNP$_{1–108}$ molecule (with no cross-reaction with BNP$_{1–32}$ or NT-proBNP$_{1–76}$). More precisely, one derived IgG1, designated mAb Hinge76, was able to recognize recombinant human proBNP$_{1–108}$ in a concentration-dependent manner, without any cross-reaction with the BNP$_{1–32}$ peptide (Fig. 1A). A minor cross-reactivity with NT-proBNP$_{1–76}$ was observed at high mAb concentrations (≥0.5 mg/L), but this was not considered significant. To identify the precise epitope recognized by mAb Hinge76, we synthesized overlapping pentadecapeptides frmeshied by 3 residues, spanning the entire sequence of human proBNP$_{1–108}$. mAb Hinge76 interacted with 6 peptides that shared the motif75PRSPKM79, corresponding to the hinge region of intact proBNP$_{1–108}$ (Fig. 1B). Western blotting experiments confirmed that the integrity of the proBNP$_{1–108}$ cleavage site (Arg$_{76}$-Ser$_{77}$) is crucial for the binding of mAb Hinge76 to proBNP$_{1–108}$; mAb Hinge76 was unable to recognize the recombinant proBNP$_{1–108}$ digested by furin (Fig. 1C, gel a, lane 2), an endoprotease having cleavage specificity for the Arg$_{76}$-Ser$_{77}$ sequence, whereas a BNP$_{1–32}$ polyclonal antibody was able to recognize BNP$_{1–32}$ after furin digestion (Fig. 1C, gel b, lane 4).

A proBNP$_{1–108}$specific immunoassay, combining mAb Hinge76 as the capture antibody with a polyclonal anti-BNP antibody as the secondary antibody, was set up to investigate the presence and the amount of the actual circulating form of proBNP$_{1–108}$ in blood samples. A typical calibration curve as well as the data for diluted plasma samples are shown in Fig. 2. The assay showed good linearity with the recombinant proBNP$_{1–108}$ in the range from 150 to 10 000 ng/L. Plasma samples diluted in the same buffer as the calibrator gave a concentration-
response curve parallel to the proBNP\textsubscript{1-108} calibration curve, suggesting that an entity immunologically indistinguishable from recombinant proBNP\textsubscript{1-108} was present in the human plasma samples. To determine the assay detection limit, defined as the lowest proBNP\textsubscript{1-108} concentration that could be differentiated from zero, we measured the calibrator diluent 20 times in a single run. The detection limit, calculated as mean/3 SD of these measurements, was 10 ng/L. The intraassay CVs were 5\%, 3.4\%, and 3\% at 500, 1000, and 5000 ng/L, respectively. At the same calibration concentrations, the interassay CVs were 6.1\%, 6.1\%, and 3.5\%, respectively. Dilution of plasma samples with high concentrations of endogenous proBNP\textsubscript{1-108} as well as mixing of plasma samples with high and low concentrations of recombinant proBNP\textsubscript{1-108} gave results within 5\% of the theoretical concentrations (data not shown). There was no significant cross-reactivity of the assay against human recombinant NT-proBNP\textsubscript{1-76} or human BNP\textsubscript{1-32} peptide introduced in a large molar excess (Table 1A). Preliminary stability experiments indicated that, at room temperature, the measured concentration of proBNP\textsubscript{1-108} at 10 h was 76\% of the initial concentration, whereas at 24 h, the measured concentration was 59\% of the original. At 4°C, plasma appeared relatively more stable with measured concentrations that were 89\% and 79\%, respectively, of the original concentration (Table 1B).

EDTA-plasma samples from 50 healthy individuals and 171 CHF patients were assayed with the proposed proBNP\textsubscript{1-108} assay. The mean proBNP\textsubscript{1-108} concentration in samples from healthy individuals was 57 ng/L (median, 54 ng/L; range, 42–99 ng/L). The concentration in the NYHA class I CHF patients was significantly increased (median, 73 ng/L) compared with the concentration in the healthy individuals (median, 54 ng/L; \(P < 0.0005\)). Mean increases of 2- to 23.5-fold were observed for the samples from patients in NYHA classes I and IV, respectively (Fig. 3A). Increases in intact proBNP\textsubscript{1-108} concentrations paralleled the disease severity, as judged by the NYHA classification. Moreover, patients for whom the LVEF value was available were grouped into 4 classes: LVEF < 30\% (n = 34), LVEF 31\%–40\% (n = 25), LVEF 41\%–50\% (n = 22), and LVEF > 50\% (n = 34). The proBNP\textsubscript{1-108} concentrations were increased in patients with decreased LVEF. When we considered all of the groups, mean proBNP\textsubscript{1-108} concentrations and LVEF were negatively correlated (Fig. 3B). For the correlation study between BNP\textsubscript{1-32} and proBNP\textsubscript{1-108} concentrations, we studied 250 samples from CHF patients. The coefficient of determination \(r^2\) between intact proBNP\textsubscript{1-108} and BNP\textsubscript{1-32} concentrations was 0.89, showing the close rela-

Fig. 1. Epitope specificity of mAb Hinge76.

(A), reactivity and specificity of mAb Hinge76 in ELISA using different immobilized proteins (proBNP\textsubscript{1-108}, NT-proBNP\textsubscript{1-76}, and BNP\textsubscript{1-32}) or an irrelevant protein (GST). mAb Hinge76 was tested at the following concentrations: 0.5 mg/L (○); 0.1 mg/L (●); 0.05 mg/L (□); 0.01 mg/L (▲); 0.005 mg/L (◆); 0.001 mg/L (◇); and 0 mg/L (■). (B), Spot method identification of peptides recognized by mAb Hinge76: panel 1, reactivity of mAb Hinge76 with 6 peptides within the proBNP\textsubscript{1-108} sequence; panel 2, sequences of reactive pentadecapeptides and identification of the minimal epitope (boxed). (C), loss of reactivity of the proBNP\textsubscript{1-108} after digestion by furin: Western blotting of recombinant proBNP\textsubscript{1-108} digested by furin (lanes 2 and 4) or undigested (lanes 1 and 3) detected by mAb Hinge76 in gel a or by a polyclonal BNP antibody in gel b.
tionship between the 2 analytes (Fig. 4). Concerning the relative ratio of proBNP₁–108 to BNP₁–32, for some of the patients, proBNP₁–108 was the predominant form; for the others, the concentration of the 2 analytes was equivalent.

Discussion
Investigation of the precise circulating forms of proBNP-derived peptides remains difficult because of the lack of reliable region-specific antibodies. We thus sought to produce a mAb that could react with only the full-length proBNP₁–108 molecule. Here we report the production of such a mAb, raised against the hinge region present only in the intact proBNP₁–108 form. More precisely, the challenge was to obtain an mAb with high affinity and whose epitope was confined near the cleavage site of proBNP₁–108 (Arg⁷⁶-Ser⁷⁷). We favored an approach using synthetic peptide immunization because the peptide sequence can be tailored until an immune response with the required specificity is obtained. One disadvantage of such

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Table 1. Cross-reactivity with relevant peptides and stability of proBNP₁–108 in the proposed assay.

<table>
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<th>Sample</th>
<th>Original proBNP₁–108 concentration, ng/L</th>
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<th>4 °C 10 h</th>
<th>22 °C 24 h</th>
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Fig. 3. Relationship between proBNP₁–108 and NYHA classification. Box-and-whisker plots show BNP₁–32 and proBNP₁–108 concentrations in the groups studied (A) and their correlation with LVEF in the CHF patients (B). The plots show the median values, 25th and 75th percentiles, and the minimum and maximum (error bars). (A), patients with CHF are shown as NYHA classes I–IV. (B), plots show the negative correlation between BNP₁–32 and proBNP₁–108 concentrations and the LVEF.

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Fig. 4. Correlation between BNP₁–32 concentrations measured by the Access BNP assay (x axis) and the proBNP₁–108 concentrations measured by the present immunoassay (y axis) in 250 plasma samples from CHF patients.
an approach is that the affinity toward the whole molecule might be too low to achieve the sensitivity required for an immunoassay. To resolve this problem, we reasoned that the length of the peptide used for the immunizations is crucial, as it must to be sufficient to adopt a conformation similar to that of the hinge region in the protein so that the anti-peptide sera could cross-react strongly with the protein. In our experience, pentadecapeptides as well as longer peptides satisfied this condition. Furthermore, both the strength of the immune response and the specificity of the induced antibodies appeared to be highly dependent on the position in the carrier (the KLH) to which the peptide was linked as well as the localization of the hinge sequence RAPRSPK within the peptide. Consequently, numerous steps were necessary to optimize the peptide design as well as the immunization protocol to obtain an mAb with the high specificity of mAb Hinge 76.

Using this mAb specific for the intact form, and a commercial anti-BNP132 antibody, we developed an immunoassay for proBNP1–108. For the detection antibody, an NT-proBNP1–76 or a BNP1–32 antibody could be used. We favored the second choice for several reasons. First, Seidler et al. (28) reported that proBNP1–108 might circulate as a trimer. This oligomerization occurs through leucine zipper-like coiled-coil motifs present at the proBNP1–108 NH$_2$ terminus. To date, all of the antibodies directed against the N-terminal region have been raised against small peptides or the monomeric form of NT-proBNP1–76 so that, depending on the localization of their epitopes, their ability to recognize the circulating trimer form remains to be established. Second, several studies have demonstrated that the integrity of the BNP1–32 ring structure, formed by a disulfide bridge between Cys$_8^{85}$ and Cys$_{102}^{105}$, is crucial for the biological activity of the hormone (29), so that use of an antibody that recognizes this native loop would, to some extent, guarantee that a putatively functional molecule is being measured. Shimizu et al. (13) proposed an immunoassay to measure proBNP1–108 by combining mAb BC203, which is specific for the COOH terminus of BNP1–32, and a polyclonal antibody specific for the NH$_2$ terminus of the precursor. However, as reported in their study, mAb BC203 may capture both proBNP1–108 as well as BNP1–32; consequently, mAb BC203 could become saturated with BNP1–32, leading to possible underestimation of the quantity of circulating proBNP1–108. On the other hand, mAb BC203 is one of the mAbs used in the Shionoria BNP IRMA (Shionogi & Co, Ltd.) (30); consequently, this test measures intact proBNP1–108 in addition to the chosen analyte. Conscious of this lack of reliable region-specific antibodies, Goetze et al. (31) preferred to quantify the total amount of precursor and its N-terminal fragment in plasma. To be certain that the same epitope present in different fragments was recognized with the same affinity when bound to the same antibody, they digested all of their samples with trypsin to release a small proBNP1–21 fragment. This small peptide could come from the intact form as well as from NT-proBNP1–76, both these forms being subsequently detected by a competitive immunoassay measuring the proBNP1–108 N-terminal epitope. On the other hand, in the innovative method proposed here, the use of mAb Hinge76, which is specific for the precursor form, allows the capture of only the intact form from plasma, ensuring for the first time an accurate determination of proBNP1–108 concentrations. Our decision to focus on a mAb specific for proBNP1–108 only was motivated by the fact that this molecule may merge the advantages of both BNP1–32 and NT-proBNP1–76. It shares the ring structure with BNP1–32, which determines the hormone activity, although no natriuretic activity has yet been shown to be associated with the precursor. In addition, like NT-proBNP1–76, it would have a longer half-life than BNP1–32, which is rapidly trapped by specific receptors. In fact, from a study in sheep plasma, it appears that proBNP1–108 might have a longer half-life than BNP1–32 in circulation (32).

Our study confirms that proBNP1–108 circulates in blood and that its cleavage to yield the BNP1–32 hormone may not be linked exclusively to the myocyte secretion process. Furthermore, it demonstrates that measurement of proBNP1–108 may have clinical value. Our assay allows the differentiation of healthy individuals from patients suffering from heart failure and the stratification of patient samples based on the severity of the heart failure. We observed some overlap between the different NYHA classes for proBNP1–108 as well as for BNP1–32 concentrations, but because of the subjective nature of the NYHA classification, this result was expected. Furthermore, proBNP1–108 concentrations and LVEF were inversely correlated in the CHF patients in our study. However, this trend should be confirmed in more homogeneous cohorts, as some individual values were far from the regression line. This could be explained by the facts that (a) the LVEF was determined by different techniques (electrocardiography, scintigraphy, and angiography) and (b) we did not have the data to divide the patients with abnormal left ventricular dysfunction into those with purely systolic or purely diastolic dysfunction.

Additional investigations are needed to improve our understanding of the physiologic meaning of the differences observed on a molar basis between proBNP1–108 and BNP1–32. Indeed, it appears that the relative ratio of proBNP1–108 to BNP1–32 varies from one patient to another, as already observed by Tateyama et al. (12). Thus, further analyses of different clinical situations appear to be necessary to determine whether the differences in the relative proportions of the various forms of natriuretic peptides reflect different stages of heart failure. Another aspect to clarify is the mechanism of proBNP1–108 clearance, which may facilitate interpretation of plasma concentrations. For example, comparisons of the quantities of NT-proBNP1–76, BNP1–32, and proBNP1–108 in the same patient with impaired renal function could be informative.
In conclusion, intact proBNP1–108, like its cleavage products, could be used as a biomarker in the diagnosis of patients with suspected CHF; however, large-scale investigations are needed to definitively clarify its possible additive clinical contribution.

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