Quantification of Pro-B-Type Natriuretic Peptide and Its Products in Human Plasma by Use of an Analysis Independent of Precursor Processing

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Background: Measurement of cardiac natriuretic peptides or their precursors in plasma appears promising in the diagnosis of heart failure. However, the currently available assays to measure pro-B-type natriuretic peptide (proBNP)-derived peptides have produced grossly discrepant results.

Methods: We treated plasma with trypsin before assay and used in the assay an antibody specific for a processing-independent epitope of human proBNP. We then determined the total concentration of proBNP and its products in healthy volunteers and heart-failure patients.

Results: The antiserum produced (no. 98192) required an intact proBNP NH2 terminus for binding and displayed a high titer, index of heterogeneity, and binding affinity, implying that the RIA was monospecific and highly sensitive. Preanalytical tryptic treatment of plasma cleaved proBNP forms to release the N-terminal 1–21 fragment. Furthermore, enzymatic treatment of plasma also was efficient in avoiding nonspecific interference from plasma proteins, making it an expedient alternative to extraction. In healthy individuals, the total proBNP concentrations increased with age from 2.0 pmol/L (range, 0–15 pmol/L; ages 51–65 years) to 22 pmol/L (range, 3–40 pmol/L; ages 66–88 years; P < 0.0001). The increase in plasma proBNP in the elderly, however, also seems to reflect the prevalence of cardiac disease. Plasma concentrations in patients with heart failure were all markedly increased [median, 89 pmol/L (range, 29–659) vs 1.0 pmol/L (range, 0–16) in age-matched controls; P < 0.0001].

Conclusions: The processing-independent analysis measures the total proBNP product irrespective of the degree of proBNP processing. The results show that proBNP and its products circulate in low picomolar concentrations in healthy individuals.

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Human B-type natriuretic peptide (BNP), a member of the cardiac natriuretic peptide family, is a 32-amino acid peptide with potent natriuretic, diuretic, and vasodilatory endocrine functions [Ref. (1); for reviews, see Refs. (2–4)]. The BNP gene is expressed predominantly in the myocytes of the failing heart, with BNP increasingly secreted into the circulation in patients with congestive heart failure (5–7). Consequently, the diagnostic use of plasma BNP measurements has been studied extensively (8–12). Increased plasma concentrations of BNP are associated with impaired function of the left ventricle, regardless of the underlying cause, and are therefore valuable in the primary diagnosis of heart failure.

The BNP gene encodes proBNP, a 108-amino acid residue precursor in which the bioactive BNP-32 sequence constitutes the COOH terminus (Fig. 1). In 1995, Hunt et al. (13) showed that fragments corresponding to the N-terminal proBNP sequence also circulate in plasma and that the concentration is increased in heart failure patients. Although the molecular heterogeneity of proBNP-derived peptides in plasma has not been clarified, chromatographic studies have suggested the presence of a high-molecular-weight proBNP peptide as well as a shorter N-terminal fragment, most likely a 1–76 fragment, in plasma from patients with congestive heart failure (13–15). Several assays directed against the N-terminal region of proBNP have now been developed (13–19), and generally, the plasma concentrations of N-terminal proBNP fragments, like bioactive BNP-32, have been reported to be increased in patients with heart failure.

Nevertheless, there remains a troublesome discrepancy between the concentrations measured by the different immunoassays of N-terminal proBNP fragments in
healthy individuals as well as in heart failure patients. Several factors are likely to account for these discrepancies. For example, differences in the epitope(s) in the precursor used by the various immunoassays are often overlooked. Moreover, plasma proteins may interfere in immunoassays, and the degree of interference may vary substantially. Consequently, some assays require extraction of plasma before analysis, which could also contribute to extraction of proBNP peptides.

The aim of the present study was to develop a processing-independent analysis (PIA) for accurate quantification of proBNP and its fragments in plasma. The PIA was independent of the degree of precursor processing into different fragments. Rather than using conventional extraction procedures, we treated plasma with a proteinase (trypsin TPCK) before measurement, thereby cleaving all proBNP peptides to the 1–21 fragment. We then measured the total proBNP product in plasma from healthy individuals of different ages and from patients with severe heart failure.

Materials and Methods

Peptides

Human proBNP 1–21, the corresponding N-terminal 1–10 sequence extended C-terminally with a tyrosyl residue for tracer preparation, and the same peptide extended C-terminally with cysteine for directional carrier coupling were custom synthesized (Cambridge Biochemical Research Ltd). An N-terminal truncated fragment of proBNP 1–21 was obtained by controlled cleavage in an automated protein sequencer (20). The purity and content of the peptides were verified by reversed-phase HPLC, amino acid analysis, and mass spectrometry.

Antiserum

An antibody directed against sequence 1–10 of human proBNP was produced using 10 mg of the 1–10 fragment extended C-terminally with cysteine and coupled to 20 mg of bovine serum albumin by the 4-maleimidobenzoyl-N-hydroxysuccinimide ester conjugation method (21). The coupled product was dissolved in 20 mL of distilled water (conjugate solution). The antigen solution (2 mL) was mixed with 3 mL of isotonic saline and emulsified with an equal volume of complete Freund’s adjuvant (The State Serum Institute) and used for the first immunization. For booster injections, 1 mL of the antigen solution was mixed with 4 mL of saline and an equal volume of incomplete Freund’s adjuvant. Eight randomly bred white Danish rabbits were immunized subcutaneously over the lower back at 8-week intervals. Fourteen days after each immunization, 20 mL of blood was collected from an ear vein, and the serum was stored at −20 °C.

Preparation of tracer

The tyrosine-extended 1–10 fragment (4.5 nmol) was iodinated by a mild chloramine-T method as described previously (22) and subsequently purified on reversed-phase HPLC (RP-300 C_{8} column; 4.6 × 220 mm; Pierce) and eluted by a linear ethanol gradient (5–30%) in 10 g/L trifluoroacetic acid. The gradient was selected to ensure separation of the nonlabeled peptide from the iodinated tracer. Fractions (1 mL) were collected at a flow rate of 1.0 mL/min. To evaluate the chromatographic separation of labeled and nonlabeled peptides, 1 mL of the monoidinated peak fraction was mixed with 10 pmol of nonlabeled tyrosine-extended proBNP 1–10 and reapplied to the HPLC column. The radioactivity and immunoreactivity were then measured. The specific tracer radioactivity was determined by self-displacement (23). Peptide calibrators were prepared from synthetic tyrosine-extended proBNP 1–10 in a Tris buffer containing 2 g/L human serum albumin (pH 8.5).

Enzymatic Treatment of Plasma

Plasma for measurement was initially treated with trypsin TPCK (Worthington Biochemical Cooperation) as described previously (24). Briefly, plasma samples and calibrators (200 μL) were mixed with 1 mL of 0.1 mol/L sodium phosphate buffer (pH 7.5) containing trypsin to a final concentration of 2 g/L trypsin in the incubation mixture. The mixture was incubated at room temperature for 30 min and then immediately boiled for 10 min to terminate the enzymatic reaction. Time intervals for the different reactions were chosen based on earlier studies (24) as well as new time and dose experiments. Trypsin-treated samples and calibrators were then stored at −80 °C. Samples were centrifuged for 10 min at 3000g before assay setup, with only the supernatant used in the subsequent RIA.

Plasma Samples

To establish a reference interval, we enrolled 90 healthy volunteers with no medical history or symptoms of systemic or cardiac disease. Volunteers were divided into two groups: ages 23–50 (n = 65; 28 females and 37 males; median age, 33 years) and ages 51–65 (n = 25; 14 females and 11 males; median age, 53 years). Blood samples were collected with volunteers in the fasting state and seated after a 20-min rest. A group consisting of 18 elderly individuals (median age, 72 years; range, 66–88 years; 10 females and 8 males) with no history of cardiac disease or symptoms were recruited from a local dancing club. Another group of 18 elderly individuals (median age, 68 years; range, 60–79 years; 9 females and 9 males) with no history of cardiac disease or symptoms and signs were assessed using echocardiography, blood pressure measurements, exercise testing (ergometry), pulmonary function tests, and blood screening; they were all without findings of cardiopulmonary disease. Finally, 16 stable heart-failure patients referred for evaluation for cardiac transplantation (median age, 55 years; range, 30–68 years; 6 females and 10 males) with no biochemical signs of renal disease were examined. The heart failure patients were classified in New York Health Association groups II–IV,
and all had a reduced left ventricular ejection fraction (median, 30%); range, 15–40%). Blood samples were collected from a cubital vein, and plasma was stored at −80 °C until analysis. The local medical ethics committee approved the use of human plasma, and informed consent was obtained from all volunteers and patients (KF01-231/99).

RIA PROCEDURE
For RIA, trypsin-treated calibrators and plasma samples (150 μL of each) were mixed with a Tris buffer (100 μL) containing 2 g/L human serum albumin, tracer peptide (1000 counts/min), and antiserum (final dilution, 1:150 000). Synthetic tyrosine-extended proBNP 1–10 was used as the calibrator. After 5 days of incubation at 4 °C, the antibody-bound and free tracer were separated by adding 2 mL of plasma-coated charcoal (15 g/L charcoal in Tris buffer containing 100 mL/L plasma), left for 10 min at room temperature, and centrifuged. Controls, buffer blanks, and sample blanks (without antiserum added) were included in the assays, and samples were always assayed in duplicate. The assay was evaluated with respect to detection limit, specificity, and imprecision (intra- and interassay).

CHROMATOGRAPHY
Plasma was applied to a Sephadex G-50 Superfine column (2000 × 10 mm; Pharmacia) and eluted at 4 °C with a Tris buffer containing 2 g/L human serum albumin at pH 8.5 (flow rate, 4 mL/h). The columns were calibrated with buffer containing 2 g/L human serum albumin at pH 8.5 °C, 1:150 000). Synthetic tyrosine-extended proBNP 1–10 was used as the calibrator. After 5 days of incubation at 4 °C, the antibody-bound and free tracer were separated by adding 2 mL of plasma-coated charcoal (15 g/L charcoal in Tris buffer containing 100 mL/L plasma), left for 10 min at room temperature, and centrifuged. Controls, buffer blanks, and sample blanks (without antiserum added) were included in the assays, and samples were always assayed in duplicate. The assay was evaluated with respect to detection limit, specificity, and imprecision (intra- and interassay).

STATISTICAL ANALYSIS
Results are expressed as medians and ranges. The reference interval was calculated by the nonparametric method of Reed et al. (25). Statistical comparisons were performed by the Mann–Whitney nonparametric test, and two-tailed P values <0.05 were considered significant.

Results

RADIOIOCIDATION
The incorporation of 125I into tyrosine-extended proBNP 1–10 varied from 80% to 95%. Labeled and nonlabeled peptides were completely separated, and the dilution curves for labeled and nonlabeled antigens were parallel. The specific activity of the tracer was estimated by self-displacement to be 3.90 Ci/μmol.

ANTISERUM EVALUATION
All rabbits responded to the immunization, and antiserum 98192 was chosen for further characterization because of its high titer (1:150 000) and binding affinity. Hence, the avidity of this antiserum, expressed by the effective equilibrium constant (K°_eff) was 0.45 × 10^{12} L/mol (26, 27), corresponding to a detection limit of 0.07 pmol/L (26). The detection limit, when calculated as the mean of 10 replicates of the zero calibrator +3 SD, was 0.20 pmol/L. The index of heterogeneity (28) was 1.10, indicating that the ligand was highly homogeneous (29) and that the antiserum acted as a solution of monoclonal antibodies (30).

The specificities of the antisera were expressed as ratios of the median inhibitory dose (ID_{50}) for the truncated N-terminal proBNP peptide in tracer displacement. Removal of the N-terminal histidine decreased the binding dramatically (ID_{50} = 0.001), and removal of two N-terminal amino acids, histidine and proline, decreased the binding further (ID_{50} = 0.0006). Consequently, the two N-terminal amino acids constitute an essential part of the epitope for antiserum 98192. The specificity was further evaluated by measuring N-terminal proBNP concentrations in heart extracts from mouse and pig (Fig. 1). No immunoreactivity was detected in either species, indicating that the amino acids in positions 5 and 6 (serine and proline) in the human sequence are necessary for antibody binding. The computer program FASTA was used to search the SwissProt database for amino acid sequences resembling human N-terminal proBNP. No relevant sequences other than the corresponding N-terminal proBNP sequences of other mammals were found.

ASSAY RELIABILITY
Dilution curves for trypsin-treated plasma samples paralleled the calibration curve (n = 3; data not shown), indicating that the affinity of the antiserum to native peptide antigen was equal to that of the synthetic peptides. The interassay CVs of replicate samples were 34% at 5 pmol/L, 20% at 16 pmol/L, 8% at 70 pmol/L, and 10% at 145 pmol/L (n = 10). The intraassay CVs were 12% at 13 pmol/L, 7% at 75 pmol/L, and 5% at 130 pmol/L (n = 10). The dilution of plasma samples with high concentrations of endogenous N-terminal proBNP as well as mixing of plasma samples with high and low concentrations of N-terminal proBNP (as determined by the present assay) produced results within 15% from the calculated concentrations. The measuring range of the assay was 0–250 pmol/L.

ANTISERUM SPECIFICITY EVALUATED BY CHROMATOGRAPHY OF PLASMA
Gel chromatography of normal plasma revealed apparent immunoreactivity eluting in the void volume. The same fraction was devoid of immunoreactivity after treatment with trypsin (Fig. 2). Chromatography of plasma from heart-failure patients displayed two peaks of immunoreactivity, but after tryptic cleavage of the fractions, only one peak was preserved [elution constant (K°_el) = 0.10; Fig. 3A]. When plasma from heart-failure patients was extracted using Sep-Pak C_{18} cartridges (Millipore Waters) before gel chromatography, the immunoreactivity eluted...
as a single peak in the same position as the peak after trypsin treatment of the fractions (Fig. 3B), but the total immunoreactivity in the peak after trypsin treatment was reduced. Finally, in gel-chromatography experiments, the immunoreactivity of trypsin-treated plasma from heart-failure patients eluted as a single peak corresponding to the position of the synthetic proBNP 1–21 calibrator ($K_D = 0.59$; Fig. 4).

Fig. 1. Amino acid sequences of the mouse, pig, and human pre-proBNP precursor (A), and illustration of proBNP and peptide fragments (B). (A), **bold** amino acids mark homology with the human sequence. The N-terminal prosequence 1–10 and the known human bioactive peptide (residues 103–134) are underlined. (B), • represent basic arginyl residues. Fragments 1–76 and 1–21 are shown, as is the bioactive BNP-32.
When left at room temperature for 24 h and then treated with trypsin, plasma from heart-failure patients did not reveal a significant decrease in endogenous N-terminal proBNP concentrations (from 127 to 105 pmol/L; n = 5). Likewise, initial trypsin treatment of plasma followed by subsequent incubation for 24 h at room temperature did not lead to degradation of endogenous peptide (from 128 to 125 pmol/L; n = 5).

N-Terminal proBNP Stability in Plasma

The concentrations of N-terminal proBNP in plasma after trypsin treatment are shown in Fig. 5. The medians differed significantly between the 23–50 and 51–65 years age groups [median, 1.0 pmol/L (range, 0–16 pmol/L; n = 65) vs 2.0 pmol/L (range, 0–15 pmol/L; n = 25); P < 0.01], as did the medians between the 51–65 and the 66–88 years age group [median, 22 pmol/L (range, 3–40 pmol/L; n = 18); P < 0.0001]. The plasma concentration in the selected group of elderly (age range, 60–79 years) without objective signs of cardiopulmonary disease was lower than that of the age-matched group of healthy elderly only asked for symptoms [median, 8 pmol/L (range, 4–28 pmol/L; n = 18) vs 22 pmol/L (range, 3–40 pmol/L; n = 18); P < 0.0005]. There was no significant concentration difference between males and females regardless of age. A histogram of the obtained concentrations revealed that the N-terminal proBNP measurements were not from a single distribution. Therefore, the reference interval was determined from individuals between 23 and 65 years of age, where the distribution was

**Fig. 2. Chromatographic profile of plasma from healthy individuals.**

(A), immunoreactivity of eluted nontreated plasma; (B), immunoreactivity in the fractions collected after treatment with trypsin.

**Fig. 3. Chromatographic profile of plasma from heart-failure patients.**

(A), plasma applied directly to the column; (B), profile for plasma initially extracted using Sep-Pak C18 cartridges. ○, untreated fractions; ●, trypsin-treated fractions.

**Fig. 4. Heart failure plasma treated with trypsin and subjected to gel filtration chromatography (●).**

The elution profile of synthetic proBNP 1–21 is also shown (○).
homogeneous. Given the non-gaussian distribution and that it was not possible to transform data to fit a gaussian distribution, the reference interval was determined by a nonparametric method using rank numbers (25), and the upper reference limit (97.5 percentile) could then be calculated as 15 pmol/L (confidence interval, 9–16 pmol/L; n = 90) for individuals 23–65 years of age.

**N-Terminal proBNP in Plasma from Heart-Failure Patients**

The plasma concentrations in heart-failure patients are shown in Fig. 6. N-Terminal proBNP concentrations were significantly increased compared with the age-matched group of healthy volunteers [median, 89 pmol/L (range, 29–659 pmol/L; n = 16)] vs 1.0 pmol/L (range, 0–16 pmol/L; n = 90); P < 0.0001] and were always higher than the upper reference limit (Fig. 6).

**Discussion**

The present study describes the development and characterization of a sequence-specific RIA and its use for PIA of proBNP and its products in human plasma. The antisera produced was of high binding affinity and homogeneity; and when it was used in combination with a moniodinated tracer, a sensitive and specific assay was obtained. We also cleaved proBNP with trypsin, thereby cleaving endogenous proBNP and its N-terminal fragments in plasma to the small 1–21 fragment. This step allows accurate quantitation of proBNP and its products irrespective of the degree of prohormone processing. Trypsin treatment of plasma samples before RIA also served as a useful alternative to extraction by abolishing nonspecific interference from plasma proteins in the RIA.

The human proBNP sequence contains several sites for possible amino acid derivatization and endoproteolytic cleavage (Fig. 1). Accordingly, the molecular heterogeneity of the circulating proBNP fragments has not been fully elucidated. Chromatographic profiles studied to date suggest that, in addition to BNP itself, only an N-terminal 1–76 fragment and intact proBNP circulate in plasma (13, 15). Moreover, the prosequence contains a leucine zipper-like sequence motif that has been reported to induce oligomerization of the N-terminal fragments of proBNP and pro-atrial natriuretic peptide (proANP) in plasma (31). This finding raises the possibility that oligomerized N-terminal proBNP fragments expose some regions, whereas others are not accessible to antibody binding. It is therefore not surprising that the plasma concentration of N-terminal proBNP fragments in healthy individuals and in heart-failure patients published to date show considerable variation [Refs. (13–19) and Table 1]. Most methods are based on immunoassays using antisera raised against the NH₂ terminus of proBNP, but precise definition of the epitope(s) has not always been reported. We established the free NH₂ terminus of human proBNP as the binding site by testing our antisera for binding to N-terminally truncated forms of human proBNP and to proBNP from other mammals with deviant N-terminal sequences. Furthermore, we found that trypsin treatment of plasma efficiently cleaves the endogenous N-terminal proBNP forms after the arginyl residue in position 21 (Fig. 4). We thus deliberately developed a PIA (32, 33) that measures the N-terminal 1–21 fragment of proBNP from all forms of N-terminal proBNP with equimolar potency. This assay corroborates that the concentrations of N-terminal proBNP fragments circulate in healthy individuals in the low picomolar range, in agreement with a few earlier reports (13, 19). The statement that N-terminal proBNP circulates in much higher concentrations than its bioactive counterpart, BNP, therefore seems premature.

Another factor of concern is the need for plasma extraction before RIA. Although many competitive assays use plasma extraction with Sep-Pak C₁₈ cartridges (Table 1), some have advocated the use of noncompetitive immunoassays (8). The apparent nonspecific interference of larger proteins in plasma can, however, be eradicated by treating samples with trypsin (Figs. 2–4). It is therefore

![Fig. 5. N-terminal proBNP in healthy individuals. Volunteers were divided into the following groups: I (age range, 23–50 years; n = 65); II (age range, 51–65 years; n = 25); III, elderly with no symptoms of cardiopulmonary disease (age range, 66–88 years; n = 18); IV, elderly with no objective findings of cardiopulmonary disease (age range, 60–79 years; n = 18).](image)

![Fig. 6. N-Terminal proBNP in heart-failure patients (●; n = 16) and in an age-matched control group (○; n = 90). One heart-failure patient had a plasma proBNP concentration of 659 pmol/L and is not shown. The dashed line indicates the calculated upper reference limit (15 pmol/L). The solid line indicates the median for the heart-failure patients.](image)
possible that the commonly observed nonspecific binding represents a region on some native plasma protein that changes after tryptic cleavage. The proBNP immunoreactivity in plasma from heart-failure patients after Sep-Pak extraction was further diminished by tryptic cleavage (Fig. 4). An explanation of this phenomenon may be that the PIA measures only small proBNP 1–21 fragments, whereas the assay without tryptic treatment allows for positive cooperation via oligomerization of the peptides.

The stability of N-terminal proBNP in plasma will also depend on the choice of epitope. Other authors have reported a loss of N-terminal proBNP immunoreactivity in plasma stored at room temperature (16, 35). Our RIA did not detect degradation of either intact N-terminal proBNP forms in plasma or trypsin-treated samples over 24 h. It therefore seems unlikely that the N-terminal proBNP sequence is degraded in plasma.

The N-terminal proBNP concentrations in plasma were measured in groups based on age and gender. We did find a minor but significant increase in plasma concentrations between age groups. For individuals <70 years of age, this was of no clinical importance for the calculation of an upper reference limit. We found no difference between genders. However, individuals >70 years of age without prior cardiac disease or symptoms did show a substantial increase in the concentrations of N-terminal proBNP in plasma (Fig. 5), which suggests that they in fact had unidentified cardiac dysfunction or other medical conditions. When we examined a second group of elderly >70 years of age without objective signs of cardiac dysfunction, we found that the N-terminal proBNP concentration was lower compared with the first group of elderly, but it still did not equal the concentration in the group of individuals <70 years of age. Larger studies investigating the correlation between age and N-terminal proBNP concentrations are needed before an age-related reference interval can be calculated. This will be important for the correct diagnostic use of proBNP measurements for heart failure in the elderly because this condition is increasingly encountered with high age. The N-terminal proBNP concentrations in the heart-failure patients were all higher than the age-matched reference limit. Therefore, the most beneficial diagnostic use of N-terminal proBNP measurements in plasma will possibly, as for BNP, be to exclude a diagnosis of cardiac impairment.

Considering the high prevalence of cardiac impairment in Western populations, measurements of proBNP in plasma is likely to obtain widespread use as a simple, sensitive, and early marker of left ventricular dysfunction. It is therefore not surprising that the diagnostics industry is launching immunoassay systems for measurement of proBNP in plasma. A promising approach is the electrochemiluminescence double-antibody “sandwich” immunoassay, which combines high sensitivity and a wide concentration range with direct measurement in plasma without sample pretreatment (17). This ECLIA/Elecsys system appears to provide fast and reliable results, but until full details of the molecular specificity and other reliability parameters are published for the ECLIA system, it is difficult to compare that assay with ours and to discuss problems and pitfalls at further length. To date, the present PIA approach with its precisely defined epitope provides a flexible and useful tool for the study of the pathobiology and physiology of proBNP and its products.

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References

### Table 1. Previously reported N-terminal proBNP plasma concentrations.

<table>
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<tr>
<th>Assay epitope(s)</th>
<th>Plasma extraction</th>
<th>Basal[^a]</th>
<th>Maximum[^b]</th>
<th>Reference</th>
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<td>ProBNP 1–13</td>
<td>Sep-Pak C, 18</td>
<td>7.6–16.2</td>
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<td>ProBNP 1–13</td>
<td>Sep-Pak C, 18</td>
<td>10.8 ± 1.3</td>
<td>236 ± 43</td>
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<td>ProBNP 1–21</td>
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<td>29.4 (12.5–74.5)</td>
<td>616 (114–2781)</td>
<td>(15)</td>
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<tr>
<td>ProBNP 1–21 and 30–38</td>
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<td>7[^c]</td>
<td>?[^c]</td>
<td>(16)</td>
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<tr>
<td>ProBNP 65–76</td>
<td>Sep-Pak C, 18</td>
<td>159 (120–245)</td>
<td>639 (386–911)</td>
<td>(18)</td>
</tr>
<tr>
<td>ProBNP 1–23</td>
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<td>1.5 (1.0–13.8)</td>
<td>314 (18–5800)</td>
<td>(19)</td>
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</table>

[^a]: Healthy individuals.
[^b]: Patients with cardiac diseases.
[^c]: ? not given.