

# Single Assay for Amino-Terminal Fragments of Cardiac A- and B-Type Natriuretic Peptides

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**Background:** High circulating concentrations of N-terminal fragments of A- and B-type natriuretic peptides (NT-proANP and NT-proBNP) identify patients with impaired cardiac function. ProANP-derived peptides are particularly sensitive to increased preload of the heart and proBNP-derived peptides to increased afterload; therefore, combining the information from the ANP and BNP systems into a single analyte could produce an assay with increased diagnostic and prognostic power.

**Methods:** We prepared a hybrid peptide containing peptide sequences from both NT-proBNP and NT-proANP (referred to as NT-proXNP) by recombinant techniques and used it to develop a RIA combining weighed concentrations of NT-proANP and NT-proBNP into a new virtual analyte, NT-proXNP. We used the novel method to measure the circulating concentrations in healthy persons and in patients with cardiac disorders. We also characterized the assay by HPLC analysis of the immunoreactive molecular forms in human plasma and serum.

**Results:** The results from the novel assay correlated well with independent home-made NT-proANP and NT-proBNP assays ( $r^2 = 0.75$ – $0.85$ ) as well with the arithmetic sum of NT-proANP and NT-proBNP ( $r^2 = 0.92$ ). Patients with valvular heart disease (VHD) and coronary artery disease (CAD) had significantly increased NT-proXNP concentrations. The areas under the curve (AUC) of the NT-proXNP assay in detecting VHD and CAD (0.961 and 0.924, respectively) were significantly

larger than the AUC of either NT-proANP (0.947 and 0.872) or NT-proBNP (0.913 and 0.782) assays. HPLC analysis showed that the novel NT-proXNP assay detects two major classes of circulating immunoreactivity corresponding to peptides derived from NT-proANP and NT-proBNP.

**Conclusions:** Our novel immunoassay mimics the physiologic signaling system working in the body by converging the information obtained from the activation of ANP and BNP into a single virtual analyte, NT-proXNP. It appears to have a diagnostic efficiency equal to or slightly better than that of individual NT-proANP or NT-proBNP assays.

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Circulating concentrations of cardiac A- and B-type natriuretic peptides (ANP and BNP)<sup>6</sup> are markers of cardiac performance and heart failure (1). Atrial and ventricular cardiomyocytes produce proANP and proBNP, which are cleaved into the biologically active ANP and BNP and their amino-terminal counterparts (NT-proANP and NT-proBNP). Increased cardiac pressure or volume as well as neurohumoral factors can trigger the synthesis and release of peptides derived from proANP and proBNP (2). Circulating concentrations of ANP and NT-proANP primarily reflect increased preload, whereas BNP and NT-proBNP concentrations primarily reflect increased afterload of the heart (1).

Both competitive and sandwich assays have been developed for the measurement of NT-proANP and NT-proBNP by use of radioactive, enzymatic, or chemiluminescence labels (3, 4), and they have been shown to be useful diagnostic and prognostic tools in heart failure and

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<sup>6</sup>Nonstandard abbreviations: ANP and BNP, A- and B-type natriuretic peptide, respectively; NT-proANP and -proBNP, N-terminal profragments of ANP and BNP, respectively; NT-proXNP, hybrid peptide of NT-proANP and NT-proBNP; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase HPLC; GF-HPLC, gel-filtration HPLC; VHD, valvular heart disease; CAD, coronary artery disease; 95% CI, 95% confidence interval; and AUC, area under the curve.

myocardial infarction (5–10). Increased plasma concentrations of peptides derived from proANP are good markers of acute overload and rapid hemodynamic changes (e.g., tachycardia), whereas proBNP-derived peptides appear to be better markers of ventricular overload (e.g., aortic stenosis) (6). Markedly increased circulating concentrations of both types of these peptides suggest combined atrial and ventricular overload (e.g., dilated cardiomyopathy) (6).

Currently available cardiac natriuretic peptide assays measure one analyte at a time (3). However, despite the fact that two distinct biologically active and independently regulated natriuretic peptides (ANP and BNP) are produced by the heart, their actions on target cells are mediated by a single receptor, natriuretic peptide receptor A (11). A major strength of the natriuretic peptides in the diagnosis of cardiac diseases lies in the high negative predictive value. Hence, an assay capable of using the information gained from both proANP- and proBNP-derived peptides would mimic the natural signaling process and could have greater clinical power. The major advantage in diagnostic use of proBNP-derived peptides over those derived from proANP appears to result from the low basal secretion rate, which provides an impressive dynamic range in pathologic situations (12); therefore, an obvious risk in a combination assay would be the masking of modestly increased proBNP peptide concentrations by the relatively high basal concentrations of proANP peptides.

The aim of this study was to develop a technically simple immunoassay that yields simultaneous information from both the ANP and BNP systems. For this purpose, we prepared recombinant hybrid peptides containing appropriate spliced sequences from NT-proBNP and NT-proANP peptides (referred to as NT-proXNP). We raised specific antisera (13) and developed a competitive immunoassay sensitive to both NT-proANP and NT-proBNP. We demonstrated the performance of our NT-proXNP assay in healthy persons and in cardiovascular patients. We also characterized the assay by analyzing by HPLC the immunoreactive NT-proXNP components in human blood.

## Materials and Methods

### PRODUCTION AND PURIFICATION OF GLUTATHIONE S-TRANSFERASE FUSION PROTEINS

We prepared the expression constructs by subcloning PCR fragments of both the ANP and BNP genes into pGEX-4T-1 vector (Amersham Pharmacia Biotech). The nucleotide sequences encoding amino acids 1–98 of human NT-proANP and 1–76 of human NT-proBNP [a construct for glutathione S-transferase (GST)-NT-proANP<sub>1–98</sub>-NT-proBNP<sub>1–76</sub>; Fig. 1A] were amplified by reverse transcription-PCR from human atrial RNA. The oligonucleotide 5' primer for NT-proANP amplification contained the cleavage site for the restriction enzyme *Bam*HI (5'-GCGGATCCAATCCCATGTACAATGC-3') and the 3'

primer for *Xba*I (5'-GCTCTAGACCGAGGGGCAGT-GAGC-3'). The 5' primer for NT-proBNP had an *Xba*I linker (5'-GCTCTAGACACCCGCTGGGCAGCCCCG-3'), the 3' primer for *Eco*RI linker, and a termination codon (5'-GCGAATTCTCATCGTGGTGCCCCGAG-3'). Alternatively, the nucleotides encoding amino acids 1–76 or 1–37 of human NT-proBNP and those encoding amino acids 1–98 or 29–98 of human NT-proANP (constructs for GST-NT-proBNP<sub>1–76</sub>-NT-proANP<sub>1–98</sub> and GST-NT-proBNP<sub>1–37</sub>-NT-proANP<sub>29–98</sub>; Fig. 1A) were amplified. The 5' primer for NT-proBNP amplification contained *Bam*HI linker (5'-GCGGATCCACCCGCTGGGCAGCCCCG-3') and the 3' primers for *Xba*I linkers (1–76, 5'-GCTCTAGATCGTGGTGCCCCGAG-3'; 1–37, 5'-GCTCTAGAGGATGTCTGCTCCACC-3'). The 5' primers for NT-proANP amplification included *Xba*I linkers (1–98, 5'-GCTCTAGAAATCCCATGTACAATGC-3'; 29–98, 5'-GCTCTAGAGAAGATGAGGTCGTGC-3'), the 3' primer for *Eco*RI linker, and a termination codon (5'-GCGAATTCTCACCGAGGGGCAGTGAGC-3'). The reverse transcription-PCR products were purified by agarose electrophoresis, cleaved with *Xba*I and *Bam*HI or *Eco*RI, and subcloned end-to-end into the *Bam*HI/*Eco*RI site of pGEX-4T-1 vector. Nucleotide sequences and reading frames of the constructs were confirmed by automated sequencing on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems).

An overnight culture of *Escherichia coli* BL21 transformed with recombinant plasmid was diluted 1:100 in 2xYTA (yeast extract, tryptone, NaCl, pH 7) and grown at 37 °C until the  $A_{660\text{ nm}}$  reached 0.6. Isopropyl-1-thio- $\beta$ -D-galactopyranoside was added to a final concentration of 0.1 mmol/L, and the culture was incubated for an additional 1–2 h. The bacterial cells were harvested by centrifugation at 7000g for 10 min at 4 °C, resuspended in phosphate-buffered saline (50  $\mu$ L/mL of culture), and sonicated. The cell lysate was cleared by centrifugation at 7000g for 15 min. The supernatant was applied to a column containing glutathione agarose (Sigma Aldrich) and washed three times with phosphate-buffered saline. The fusion protein was eluted with 10 mmol/L glutathione in 50 mmol/L Tris-HCl, pH 8.0 (14), and stored in aliquots at –20 °C. The products were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

### PREPARATION AND PURIFICATION OF RECOMBINANT NT-proXNP

Recombinant NT-proXNP (NT-proBNP<sub>1–37</sub>-NT-proANP<sub>29–98</sub>) was released from the GST fusion partner by treatment with thrombin at room temperature for 1 h (10 U/mg of protein). The peptide was purified by reversed-phase HPLC (RP-HPLC) on a 1 (i.d.)  $\times$  25 cm Vydac C<sub>4</sub> column (Separations Group), eluted with a linear 30-min gradient from 20% to 48% acetonitrile in aqueous trifluoroacetic acid (1 mL/L trifluoroacetic acid in water). The elution rate was 1 mL/min, and the absorbance at 214–280 nm

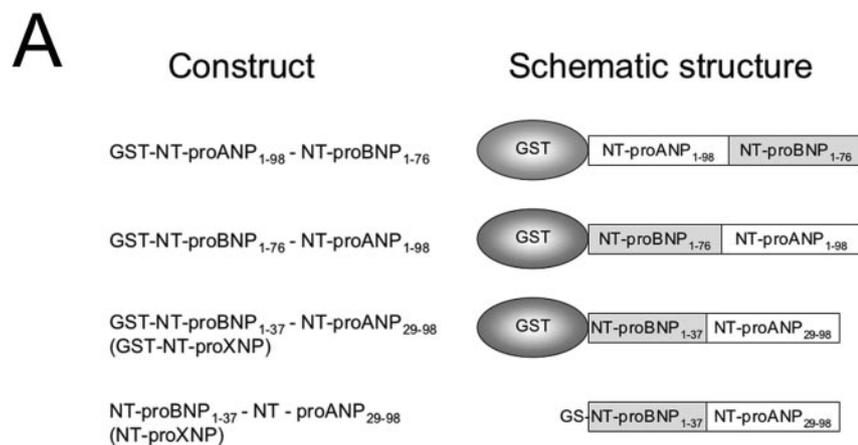
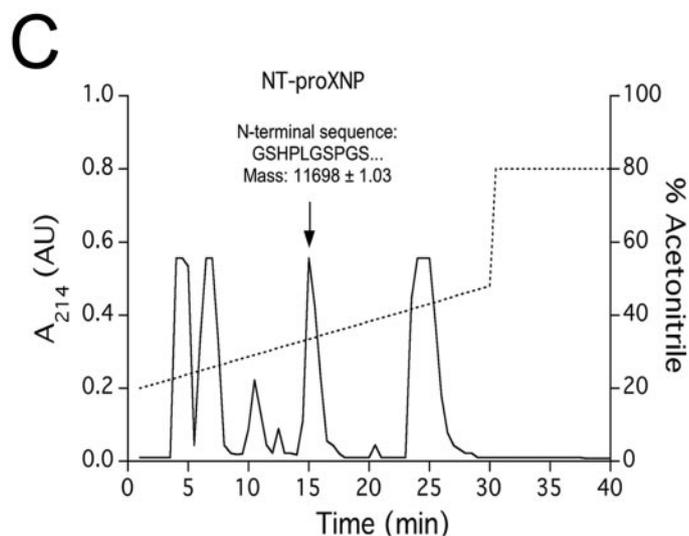
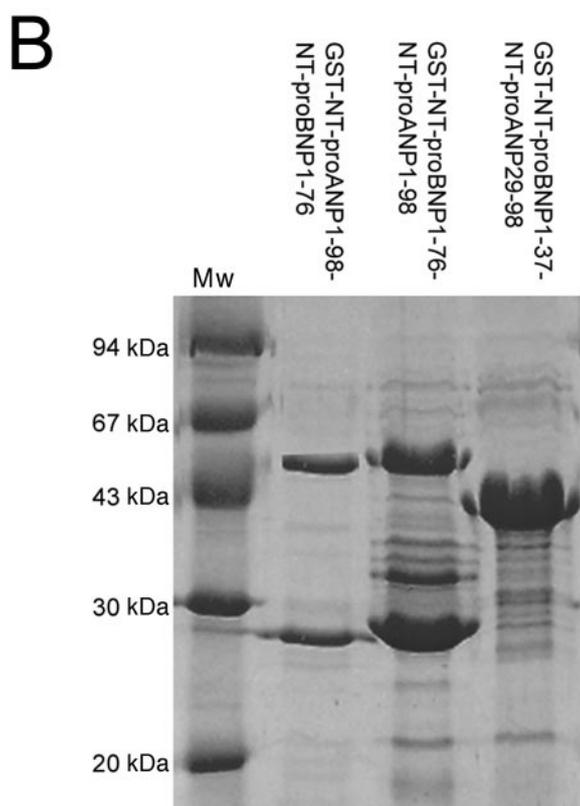


Fig. 1. Purification and characterization of GST-fusion proteins and recombinant NT-proXNP.

(A), schematic structures of the recombinant proteins GST-NT-proANP<sub>1-98</sub>-NT-proBNP<sub>1-76</sub>, GST-NT-proBNP<sub>1-76</sub>-NT-proANP<sub>1-98</sub>, GST-NT-proBNP<sub>1-37</sub>-NT-proANP<sub>29-98</sub> (GST-NT-proXNP), and NT-proBNP<sub>1-37</sub>-NT-proANP<sub>29-98</sub> (NT-proXNP). (B), SDS-PAGE analysis of the GST-fusion proteins. Lane Mw, molecular markers. (C), RP-HPLC analysis of affinity-purified recombinant NT-proXNP from which the GST partner was released with thrombin digestion. AU, absorbance units.



was measured during HPLC to monitor the purity of products. The peptide was quantified by gel-filtration HPLC (GF-HPLC) with the absorbance at 214 nm measured against a weighed desiccated bovine serum albumin calibrator. GF-HPLC was performed on a Waters ProteinPak-125 [3.9 (i.d.) × 300 mm] column with a mobile phase of 400 mL/L acetonitrile in aqueous trifluoroacetic acid (1 mL/L trifluoroacetic acid in water) and a flow rate of 1 mL/min. The amino-terminal sequence of recombinant NT-proXNP was confirmed by 10 cycles of automated Edman degradation on an ABI 477A gas-phase sequencer (Applied Biosystems). The molecular mass was determined by mass spectrometry on a QToF I-ES mass spectrometer (Micromass Ltd.).

#### PEPTIDE SYNTHESIS

Synthetic peptides (NT-proANP<sub>1-20</sub>, NT-proANP<sub>39-59</sub>, NT-proANP<sub>60-80</sub>, NT-proANP<sub>70-90</sub>, NT-proBNP<sub>1-22</sub>, NT-proBNP<sub>10-29</sub>, NT-proBNP<sub>52-70</sub>, and Tyr-NT-proBNP<sub>57-76</sub>) were assembled by use of Fmoc chemistry on an ABI 433A Peptide Synthesizer (Applied Biosystems).

#### ANTISERA

The affinity-purified GST/NT-proANP fusion protein was used as such as an immunogen. Before immunization, the chemically synthesized peptides (NT-proANP<sub>1-20</sub>, NT-proANP<sub>39-59</sub>, NT-proANP<sub>60-80</sub>, NT-proBNP<sub>1-22</sub>, NT-proBNP<sub>10-29</sub>, NT-proBNP<sub>52-70</sub>, and Tyr-NT-proBNP<sub>57-76</sub>) were coupled by water-soluble carbodiimide to bovine

thyroglobulin or horseshoe crab hemocyanin (Sigma Aldrich). Goats received injections at multiple sites in the back with 1.0 mg of immunogen emulsified in an equal volume of Freund's complete adjuvant (Difco Laboratories). Boosters of 0.5 mg in Freund's incomplete adjuvant were given two to four times at 2- to 3-week intervals, and the goats were bled 14 days after the injections.

#### TRACER PREPARATION

Recombinant NT-proXNP (140 pmol) was radioiodinated by use of 0.5 mCi of Na<sup>125</sup>I in the presence of 10 µg of chloramine-T in 0.5 mol/L phosphate buffer (pH 7.5) for 1 min, followed by addition of 10 µg of sodium disulfite. The reaction mixture was purified by Sephadex G-25 (Amersham Pharmacia Biotech) gel-filtration chromatography followed by RP-HPLC on a Symmetry C<sub>18</sub> column [4.6 (i.d.) × 150 mm; Waters]. The column was eluted with a 30-min gradient of 20–50% acetonitrile in aqueous trifluoroacetic acid (1 mL/L trifluoroacetic acid in water) at a flow rate of 1 mL/min. Fractions (1-mL) were collected and monitored for radioactivity in a MultiGamma counter (Wallac). Iodinated peptide was usable for at least 5 weeks when stored in aliquots at –20 °C.

#### RIA PROCEDURE

All calibrators, sample dilutions, and antiserum solutions were made up in RIA assay buffer (0.04 mol/L sodium hydrogen phosphate, 0.01 mol/L sodium dihydrogen phosphate, 0.1 mol/L NaCl, 1 g/L gelatin, 0.5 mL/L Triton X-100, pH 7.4). Calibrators (80–8000 pmol/L recombinant NT-proXNP) or plasma or serum samples were pipetted in 25-µL duplicates and incubated with 100 µL of antiserum solution [a combination of anti-NT-proANP (final dilution, 1:14 000) and anti-NT-proBNP (final dilution, 1:34 000)] together with 100 µL of tracer solution containing 8000–10 000 cpm of the iodinated peptide for 16–24 h at 4 °C. The bound and free peptides were separated by precipitation with 0.5 mL of polyethylene glycol 6000 (80 g/L) containing normal goat serum as a carrier and donkey anti-goat IgG (Linco Research, Inc.). After centrifugation, the pellet was counted for radioactivity (Clinigamma 1272; Wallac).

#### RIA VALIDATION

Serial dilutions of human plasma and serum were assayed against the NT-proXNP calibrator to test for parallelism. We assessed the linearity of the assay by linear regression analysis of results obtained for a normal plasma pool to which recombinant NT-proANP and NT-proBNP were added (100–7500 pmol/L; n = 4 each). We assessed within-assay imprecision by measuring 10 replicates of three pooled (from different specimens) plasma or sera with different concentrations of NT-proXNP. The same samples were also used for the between-assay precision studies (n = 10). We determined recoveries by measuring serum pools to which 200, 1500, or 4000

pmol/L NT-proANP and/or NT-proBNP had been added (n = 10 each). The differences between measured total and endogenous peptide were expressed as a percentage of the added peptide. We studied the stability of the analyte in serum by storing 10 patient samples with various concentrations of immunoreactive NT-proXNP at –20 and 4 °C for 11 days and at 22 °C for 3 days. NT-proXNP was measured on days 1, 3, 6, and 11, and the result was compared with the initial concentration of each individual sample.

#### OTHER RIAs

NT-proBNP (antiserum to NT-proBNP<sub>10–29</sub>) and NT-proANP (antiserum to NT-proANP<sub>46–79</sub>) concentrations were measured by RIAs as described previously (13).

#### HPLC ANALYSIS OF HUMAN PLASMA

A human plasma or serum sample (300 µL) was mixed with 200 µL of 1 mL/L trifluoroacetic acid in acetonitrile and cleared by centrifugation at 10 000g for 30 min. The supernatant was passed through a 0.45 µm Millipore filter, and the filtrate was subjected to RP- or GF-HPLC analysis. For RP-HPLC, the sample was applied to a Vydac C<sub>4</sub> [4.6 (i.d.) × 150 mm] column and eluted with a linear 40-min gradient from 16% to 40% acetonitrile in 1 mL/L aqueous trifluoroacetic acid. The flow rate was 1 mL/min, and 1-mL fractions were collected. For GF-HPLC analysis, the sample was applied to a Proteinpak 125 column [3.9 (i.d.) × 300 mm; Waters] and eluted with 400 mL/L acetonitrile in aqueous trifluoroacetic acid (1 mL/L trifluoroacetic acid in water). The flow rate was 1 mL/min, and 0.5-mL fractions were collected. The chromatography fractions were dried in a SpeedVac concentrator and dissolved in 0.5 mL of RIA assay buffer for use in the RIAs.

#### PATIENTS AND SAMPLE COLLECTION

Venous serum samples were collected from 100 healthy blood donors (age range, 20–65 years; 50% males) into Vacutainer Tubes. Serum samples were obtained from 111 patients with valvular heart disease (VHD; age range, 29–81 years; 51% women). Forty-two (38%) of the VHD patients had aortic valvular disease, 57 (51%) had mitral valvular disease, and 12 (11%) had both aortic and mitral valvular disease. Serum samples were also obtained from 367 patients with coronary artery disease (CAD; age range, 33–88 years; 30% women). Twenty-eight (8%) of the CAD patients had postinfarction angina (1 week after acute myocardial infarction), and 160 (44%) had previous acute myocardial infarction. Of the 478 patients with VHD or CAD, 355 (74%) had hypertension, 146 (31%) had diabetes mellitus, and 304 (64%) had dyslipidemia. The medical therapies used before hospitalization included beta-blockers (76% of patients), angiotensin-converting enzyme inhibitors (69%), calcium channel blockers (21%), diuretics (24%), statins (53%), organic nitrates (53%), antithrombotic drugs (64%), and digitalis glycosides

(6%). EDTA-plasma samples for analytical studies were obtained from a subset of the patients and from healthy individuals. Ejection fractions were determined for 477 patients (111 with VHD and 366 with CAD) with M-mode echocardiography using the equation:  $EF = (SV/EDV) \times 100$ , where SV is stroke volume and EDV is end-diastolic volume. The Ethics Review Board of Semmelweis University (Budapest, Hungary) approved the clinical protocols. The investigation conformed to the principles outlined in the Declaration of Helsinki, and informed consent was obtained from all volunteers and patients. The plasma and serum samples were stored at  $-20^{\circ}\text{C}$  until analyses.

#### STATISTICAL ANALYSIS

Comparisons between two groups were performed by nonpaired nonparametric analysis (Mann-Whitney test). Statistical significance was defined as  $P < 0.05$ . Correlation coefficients were calculated by linear regression analysis. The assays were compared by use of ROC analyses (MedCalc and Graphpad Prism software). The optimal cutoff values were chosen by ROC analysis and defined as the concentration with the largest sum of sensitivity plus specificity for each analyte and clinical group.

### Results

#### PURIFICATION AND CHARACTERIZATION OF RECOMBINANT NT-proXNP

The cDNAs for the coding sequences of human NT-proBNP<sub>1-76</sub> and NT-proANP<sub>1-98</sub> as well as NT-proBNP<sub>1-37</sub> and NT-proANP<sub>29-98</sub> were inserted end-to-end into the multiple cloning sites of a bacterial expression vector pGEX-4T-1 (Fig. 1A). The GST fusion partner was placed at the amino terminus, allowing purification by affinity chromatography (14). A yield of 0.3–0.6 mg of soluble affinity-purified GST-NT-proANP<sub>1-98</sub>-NT-proBNP<sub>1-76</sub> or GST-NT-proBNP<sub>1-76</sub>-NT-proANP<sub>1-98</sub> was typically obtained from a 400-mL bacterial culture, but the proteins were highly degraded, as detected by SDS-PAGE (Fig. 1B). The affinity-purified GST-NT-proBNP<sub>1-37</sub>-NT-proANP<sub>29-98</sub> fusion protein appeared as a single band in SDS-PAGE without any major contaminants (Fig. 1B), and the yield from a 400-mL bacterial culture was typically 1 mg. According to SDS-PAGE, the size of the protein was  $\sim 41$  kDa, which is consistent with that expected from the nucleotide sequence. Because of the better yield and stability, GST-NT-proBNP<sub>1-37</sub>-NT-proANP<sub>29-98</sub> was chosen for assay development.

Recombinant human NT-proBNP<sub>1-37</sub>-NT-proANP<sub>29-98</sub> (referred to as NT-proXNP) was released from its fusion partner by digestion with thrombin. Two extra amino acids, Gly and Ser originating from the cloning vector, were left at the amino terminus of the recombinant peptide. In addition, a Ser-Arg spacer was inserted between the NT-proBNP and NT-proANP amino acid sequences. The recombinant peptide was further purified and separated from its GST partner, thrombin, and other contaminants by RP-HPLC (Fig. 1C) with a yield of 0.5

mg/L of original bacterial culture. The size of the peptide was  $\sim 12$  kDa as detected by GF-HPLC, corresponding to that expected from the amino acid sequence. The integrity of the structure of recombinant NT-proXNP was further examined by mass spectrometry and N-terminal sequencing. The mass (11 698) and the first 10 N-terminal amino acids (GSHPLGSPGS) were consistent with the expected structure of NT-proXNP (Fig. 1C), including the vector-derived N-terminal Gly-Ser dipeptide and the Ser-Arg spacer between the NT-proBNP- and NT-proANP-derived sequences. The material of this single HPLC peak was used for assay development.

#### PRODUCTION OF ANTISERA AND DEVELOPMENT OF IMMUNOASSAY

Goats were immunized with the GST-fusion proteins of NT-proANP and NT-proBNP as well as with synthetic peptide fragments (NT-proANP<sub>1-20</sub>, NT-proANP<sub>39-59</sub>, NT-proANP<sub>60-80</sub>, NT-proBNP<sub>1-22</sub>, NT-proBNP<sub>10-29</sub>, NT-proBNP<sub>52-70</sub>, and Tyr-NT-proBNP<sub>57-76</sub>) coupled to thyroglobulin or hemocyanin. After boosters (two to four), test bleeds showed 40% binding of the radioiodinated NT-proANP, NT-proBNP, or NT-proXNP at a final dilution of 1:10 000–1:50 000. Antisera to NT-proBNP<sub>10-29</sub> and NT-proANP<sub>46-79</sub> (raised against the GST-fusion protein of NT-proANP) were optimal for NT-proXNP immunoassay development. Cross-reactivities of the antisera relative to human ANP, rat ANP, human BNP, human C-type natriuretic peptide, rat adrenomedullin, and human endothelin-1 were  $< 0.03\%$ . The NT-proANP antiserum cross-reacted 100% with proANP purified from human atria but did not recognize recombinant human NT-proBNP<sub>1-76</sub>. Likewise, the NT-proBNP antiserum cross-reacted 100% with recombinant proBNP but did not recognize recombinant human NT-proANP<sub>1-98</sub> ( $< 0.03\%$ ).

Recombinant NT-proXNP was used as a calibrator, and it was also radioiodinated. In the NT-proXNP assay, NT-proANP and NT-proBNP present in human blood compete with the NT-proXNP tracer for binding of specific antisera. Different amounts of serum or plasma from healthy individuals and heart failure patients displaced the tracer in parallel with the recombinant NT-proXNP calibrator. The NT-proXNP assay was highly linear in the range 150–7300 pmol/L ( $r = 0.994$ ). At 80 pmol/L, displacement of the tracer was 5%; this was considered the detection limit of the assay. The concentration displacing 50% of the labeled NT-proXNP was 1157 pmol/L. The within-assay imprecisions (CVs) were  $\leq 6\%$ , and the between-assay imprecisions were  $\leq 12\%$ . The recoveries of the analytes varied between 61% and 90%, depending on the ratio and the concentrations of NT-proANP and NT-proBNP (Table 1). Storage of serum samples at  $-20^{\circ}\text{C}$  for 11 days, or at  $22^{\circ}\text{C}$  for 3 days, did not cause any significant change in the concentration of NT-proXNP compared with the initial concentration [linear regression analysis,  $r = 0.21$ ; slope = 0.006; 95% confidence interval

**Table 1. Recovery and reproducibility of the NT-proXNP RIA.**

Recovery experiments (n = 7)			
NT-proANP in serum, pmol/L	NT-proBNP in serum, pmol/L	Mean (SD) amount detected, pmol/L	Mean (SD) recovery, %
200	200	256 (26)	64 (6.4)
1500	200	1037 (100)	61 (5.9)
4000	200	2615 (351)	62 (8.3)
200	1500	1078 (106)	63 (6.2)
200	4000	3339 (320)	80 (7.6)
1500	1500	2443 (260)	81 (8.6)
4000	1500	3956 (128)	72 (2.3)
1500	4000	4413 (270)	80 (4.9)
4000	4000	7174 (344)	90 (4.3)
Reproducibility experiments (n = 10)			
	Pool 1	Pool 2	Pool 3
Within-assay			
Mean (SD), pmol/L	472 (28)	1769 (97)	5984 (282)
CV, %	6.0	5.5	4.7
Between-assay			
Mean (SD), pmol/L	494 (52)	1794 (224)	5890 (645)
CV, %	10	12	11

(95% CI),  $-0.00805$  to  $0.02031$  for storage at  $-20^{\circ}\text{C}$ ;  $r = 0.31$ ; slope =  $0.010$  (95% CI,  $-0.0001$  to  $0.02049$ ) for storage at  $4^{\circ}\text{C}$ ;  $r = 0.20$ , slope =  $-0.019$  (95% CI,  $-0.0651$  to  $0.02647$ ) for  $22^{\circ}\text{C}$ ;  $P > 0.05$  for all three]. We observed no significant difference in NT-proXNP concentrations between serum and EDTA plasma (Deming regression slope serum vs plasma,  $0.986$ ; 95% CI,  $0.822$ – $1.150$ ;  $n = 20$ ).

#### HPLC ANALYSES OF BLOOD SAMPLES

We used RP-HPLC to characterize the molecular forms of the immunoreactivity detected by the novel NT-proXNP assay in human circulation. Two separate areas of NT-proXNP immunoreactivity were detectable in human plasma and serum. The first, broad peak, apparently comprising several peptides, eluted before the recombinant NT-proBNP<sub>1-76</sub> calibrator and thus probably represents truncated NT-proBNP (Fig. 2A). The second immunoreactive peak eluted at the same position as the recombinant NT-proANP<sub>1-98</sub> calibrator and thus probably represents intact or nearly intact NT-proANP (13). One broad immunoreactive peak detectable in plasma and serum by GF-HPLC represented both immunoreactive NT-proANP and NT-proBNP (Fig. 2B). Thus, our NT-proXNP assay appears to recognize all components of proANP and proBNP in human blood detected by the individual NT-proANP<sub>46-79</sub> and NT-proBNP<sub>10-29</sub> assays. Very little, if any, immunoreactive material corresponding to proANP or proBNP was detectable in the chromatograms despite the fact that the antisera cross-reacted fully with the full prohormones.

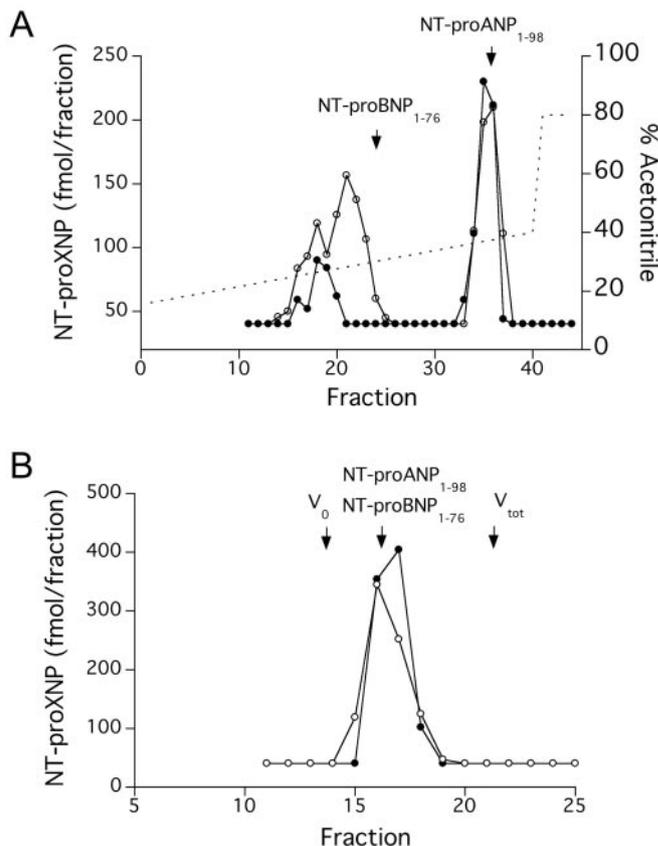


Fig. 2. RP-HPLC (A) and GF-HPLC (B) analysis of plasma (●) and serum (○) samples from different patients with acute myocardial infarction. The fractions were dried and subjected to NT-proXNP RIA. Recombinant NT-proANP<sub>1-98</sub>, NT-proBNP<sub>1-76</sub>, and proBNP<sub>1-108</sub>, as well as purified rat proANP, bovine serum albumin, and  $^{125}\text{I}$  were used for calibration of the columns (arrows).  $V_0$ , void volume;  $V_{\text{tot}}$ , total column volume.

#### NT-proXNP CONCENTRATIONS IN HEALTHY INDIVIDUALS

We analyzed serum samples from healthy individuals with the NT-proXNP, NT-proANP, and NT-proBNP immunoassays (Fig. 3 and Table 2). The median (2.5–97.5 percentiles) NT-proXNP concentration in women [ $148$  ( $<80$  to  $295$  pmol/L;  $n = 50$ ; NT-proXNP was undetectable in 5 samples)] was significantly higher than in men [ $114$  ( $<80$  to  $298$ ) pmol/L;  $n = 50$ ; 10 samples with undetectable NT-proXNP;  $P = 0.015$ ]. The NT-proANP concentration was  $249$  ( $96$ – $550$ ) pmol/L in healthy women and  $228$  ( $110$ – $473$ ) pmol/L in men. The corresponding NT-proBNP concentrations were  $88$  ( $<40$  to  $168$ ) pmol/L in women and  $80$  ( $41$ – $121$ ) pmol/L in men. We found no statistically significant gender differences in NT-proANP or NT-proBNP. It is notable that the lower the peptide concentrations were, the greater the divergence of the NT-proXNP value was from the simple arithmetic sum of the concentrations of NT-proANP and NT-proBNP (Table 1). Thus, the reference NT-proXNP concentrations in both women and men were significantly lower ( $P < 0.0001$ ) than the reference NT-proANP concentrations but were significantly higher than the NT-

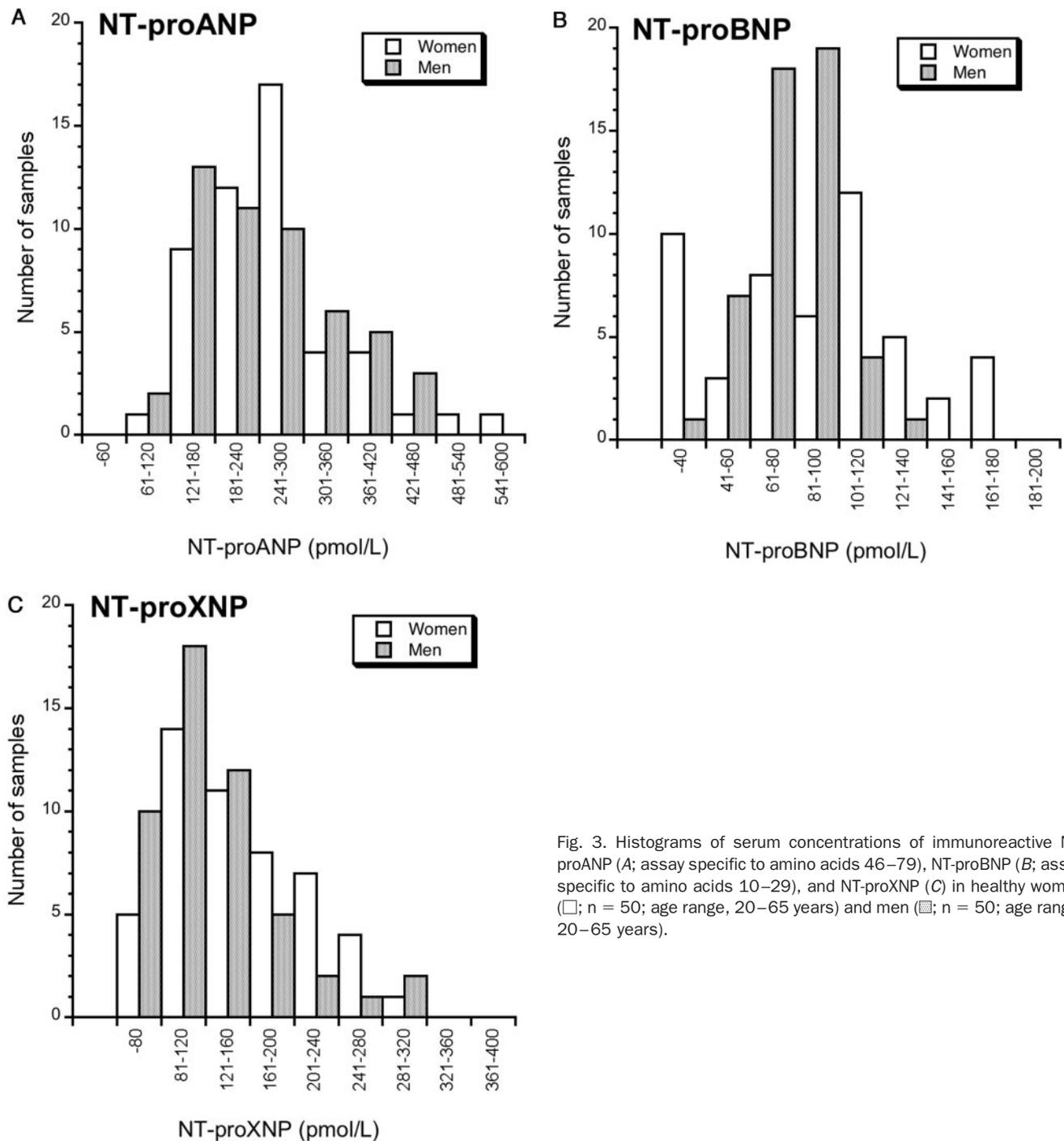


Fig. 3. Histograms of serum concentrations of immunoreactive NT-proANP (A; assay specific to amino acids 46–79), NT-proBNP (B; assay specific to amino acids 10–29), and NT-proXNP (C) in healthy women (□; n = 50; age range, 20–65 years) and men (■; n = 50; age range, 20–65 years).

proBNP ( $P < 0.0001$ ) concentrations. This is attributable to our deliberate choice of an antiserum with an affinity that would produce an NT-proXNP assay favoring NT-proBNP at the expense of NT-proANP at low peptide concentrations.

#### NT-proXNP CONCENTRATIONS IN PATIENTS WITH CARDIAC DISEASE

We used the new NT-proXNP assay and previously developed in-house assays for NT-proANP and NT-

proBNP to analyze 478 serum samples from patients with VHD (n = 111) or CAD (n = 367). The median NT-proXNP concentration was significantly higher in the VHD patients (1113 pmol/L;  $P < 0.0001$ ) than in the CAD patients (445 pmol/L; Table 2). Both values were also significantly higher than the reference values ( $P < 0.0001$ ). We observed no gender differences in either the VHD or CAD group. The NT-proXNP concentrations in both patient groups correlated well with both NT-proANP ( $r^2 = 0.75$ ) and NT-proBNP ( $r^2 = 0.85$ ). The correlation was

**Table 2. Comparison of NT-proXNP, NT-proANP, and NT-proBNP concentrations in healthy persons and in patients with VHD or CAD.**

	NT-proXNP	NT-proANP	NT-proBNP
Healthy women, <sup>a</sup> pmol/L (n = 50)	148 (102–206) <sup>b</sup>	249 (196–292)	88 (55–117)
Healthy men, <sup>a</sup> pmol/L (n = 50)	114 (82–148)	228 (169–315)	80 (69–96)
VHD patients, <sup>a</sup> pmol/L (n = 111)	1113 (553–1963)	1181 (702–1648)	421 (200–867)
Best VHD cutoff, <sup>c</sup> pmol/L	303	416	169
Mean (95% CI) sensitivity, %	88 (81–94)	89 (82–94)	79 (71–86)
Mean (95% CI) specificity, %	100 (96–100)	94 (87–98)	100 (96–100)
CAD patients, <sup>a</sup> pmol/L (n = 367)	445 (262–782) <sup>d</sup>	563 (364–884) <sup>d</sup>	177 (90–355) <sup>d</sup>
Best CAD cutoff, <sup>c</sup> pmol/L	248	408	127
Mean (95% CI) sensitivity, %	78 (73–82)	69 (64–74)	64 (59–69)
Mean (95% CI) specificity, %	95 (89–98)	93 (86–97)	91 (84–96)
Patients separated by left ventricular function <sup>a</sup>			
LVEF <sup>e</sup> >50%, pmol/L (n = 370)	465 (265–860)	582 (369–966)	179 (93–364)
LVEF = 41–50%, pmol/L (n = 64)	666 (35–1324) <sup>f</sup>	758 (438–1335) <sup>g</sup>	283 (137–563) <sup>f</sup>
LVEF = 31–40%, pmol/L (n = 30)	978 (614–1822) <sup>g</sup>	1154 (624–1763) <sup>g</sup>	405 (258–805) <sup>f</sup>
LVEF ≤30%, pmol/L (n = 13)	2418 (1230–3834) <sup>f</sup>	1585 (1264–2198) <sup>g</sup>	1105 (548–1635) <sup>f</sup>

<sup>a</sup> Median (25th–75th percentiles) values.<sup>b</sup>  $P < 0.05$  vs healthy men.<sup>c</sup> Obtained from ROC curves by comparison with healthy controls.<sup>d</sup>  $P < 0.0001$  vs VHD.<sup>e</sup> LVEF, left ventricular ejection fraction.<sup>f,g</sup> Compared with the preceding left ventricular functional group: <sup>f</sup> $P < 0.01$ ; <sup>g</sup> $P < 0.05$ .

even better between NT-proXNP and the arithmetic sum of NT-proANP and the NT-proBNP ( $r^2 = 0.92$ ). The linear correlation for the NT-proANP and NT-proBNP concentrations was markedly lower ( $r^2 = 0.51$ ). Thus, the NT-proXNP assay can simultaneously detect activation of the ANP and BNP systems regardless of whether they are activated alone or together.

Using ROC analysis, we tested the usefulness of increased serum concentration of NT-proXNP as an indicator of cardiac disease (Fig. 4). The mean (95% CI) area under the curve (AUC) for detecting patients with VHD was 0.961 (0.933–0.989; Fig. 4A), and for detecting patients with CAD was 0.924 (0.900–0.947; Fig. 4B). The corresponding AUC for NT-proANP were 0.947 (0.913–0.981) and 0.872 (0.840–0.904), and for NT-proBNP were 0.913 (0.870–0.956) and 0.782 (0.742–0.823), respectively. Statistical comparison showed that the AUC of NT-proXNP was significantly larger than that of NT-proBNP in patients with VHD ( $P = 0.004$ ) and larger than those of both NT-proANP and NT-proBNP in patients with CAD ( $P < 0.0001$ ). We also performed ROC curve analysis for the sum of the NT-proANP and NT-proBNP concentrations. The AUC for the VHD patients was 0.947 (0.913–0.981), and for the CAD patients was 0.875 (0.844–0.906). The best cutoff values for detecting VHD and CAD and the corresponding sensitivities and specificities are shown in Table 2.

We also examined whether our assays can detect impaired systolic ventricular function characterized by ejection fraction ≤50% (Fig. 4C). The AUC values for NT-proXNP (0.689; 95% CI, 0.631–0.747), NT-proANP (0.672; 0.612–0.733), NT-proBNP (0.684; 0.624–0.744), and

the arithmetic sum of NT-proANP and NT-proBNP (0.686; 0.626–0.746) did not differ significantly from each other ( $P > 0.05$ ). We further divided the patients into four different groups according to the ejection fraction (≤30%, 31–40%, 41–50%, and >50%), and the assays were again equal in distinguishing among the groups (Table 2).

## Discussion

The ANP and BNP peptide systems diverge in terms of regulation of gene expression and peptide secretion; thus, their plasma concentrations differ markedly in different pathologic conditions (6). Despite this, the signaling functions of ANP and BNP to the target cells are mediated by a common receptor, natriuretic receptor A (11). We therefore hypothesized that an assay that can combine the information derived from changes in both the ANP and BNP systems might have the advantage of higher clinical sensitivity over that provided by any individual assay. To test this hypothesis, we developed a simple combined immunoassay for the simultaneous measurement of circulating NT-proANP and NT-proBNP.

We prepared several hybrid combination epitope peptides that contained amino acid sequences from both NT-proANP and NT-proBNP in a single molecule by recombinant techniques in *E. coli*. However, the GST-fusion proteins containing the full-length NT-proBNP and NT-proANP sequences (GST-NT-proANP<sub>1–98</sub>-NT-proBNP<sub>1–76</sub> or GST-NT-proBNP<sub>1–76</sub>-NT-proANP<sub>1–98</sub>) showed major degradation during the expression procedure. This may be related to the fact that the sequences of proANP and proBNP contain leucine zipper-like motifs, which might induce oligomerization (15). Moreover, NT-

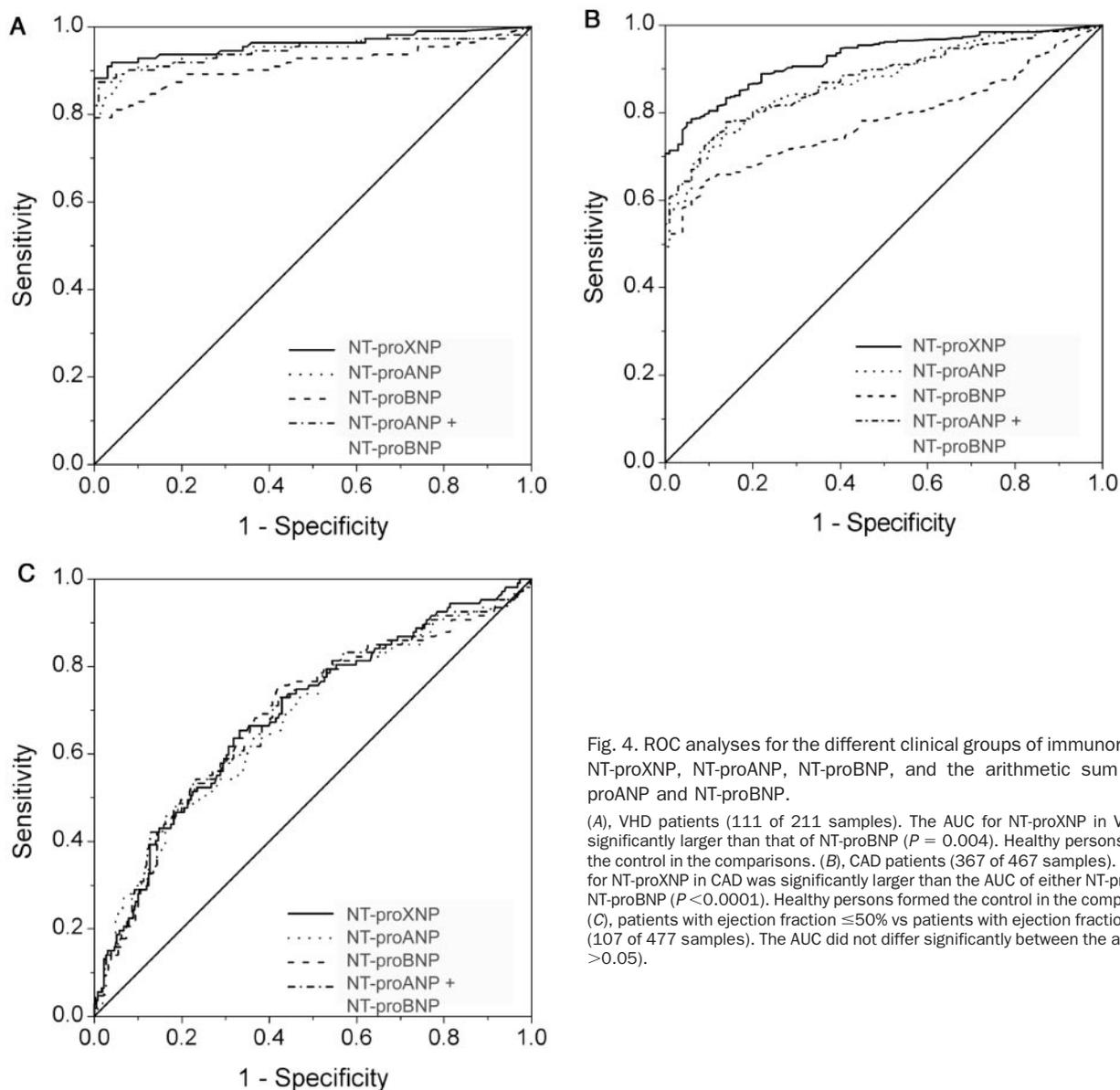


Fig. 4. ROC analyses for the different clinical groups of immunoreactive NT-proXNP, NT-proANP, NT-proBNP, and the arithmetic sum of NT-proANP and NT-proBNP.

(A), VHD patients (111 of 211 samples). The AUC for NT-proXNP in VHD was significantly larger than that of NT-proBNP ( $P = 0.004$ ). Healthy persons formed the control in the comparisons. (B), CAD patients (367 of 467 samples). The AUC for NT-proXNP in CAD was significantly larger than the AUC of either NT-proANP or NT-proBNP ( $P < 0.0001$ ). Healthy persons formed the control in the comparisons. (C), patients with ejection fraction  $\leq 50\%$  vs patients with ejection fraction  $> 50\%$  (107 of 477 samples). The AUC did not differ significantly between the assays ( $P > 0.05$ ).

proBNP antisera bound recombinant NT-proANP<sub>1-98</sub>–NT-proBNP<sub>1-76</sub> only weakly. We therefore designed a construct encoding NT-proBNP<sub>1-37</sub>–NT-proANP<sub>29-98</sub> in which the leucine zipper-like motif regions were not present. This peptide, referred to as NT-proXNP, was more stable and easier to express. It was also recognized by the NT-proANP and NT-proBNP antisera with avidity similar to the avidity for recombinant NT-proANP<sub>1-98</sub> and NT-proBNP<sub>1-76</sub>, respectively.

Using this novel NT-proXNP peptide and well-characterized antisera for NT-proANP and NT-proBNP (13), we developed a RIA for the simultaneous detection NT-proANP and NT-proBNP. The concentrations of both NT-proANP and NT-proBNP can be measured directly in a small amount of plasma or serum without previous extraction. In this simple assay format, NT-proANP and

NT-proBNP peptides present in human blood samples compete with the NT-proXNP tracer for binding to the specific antisera to NT-proANP and NT-proBNP. Thus, a single laboratory value reflects the activation or inactivation of the ANP or BNP systems or both. The assay was designed, by the selection of antiserum affinity, to give more weight to NT-proBNP over NT-proANP at low peptide concentrations. This compensates for the potential diagnostic disadvantage of the relatively high basal secretion of NT-proANP. Nevertheless, the results of the novel NT-proXNP assay correlated well with the results obtained by individual NT-proANP and NT-proBNP assays and even better with the arithmetic sum of NT-proANP and NT-proBNP.

We partially characterized by HPLC the components in human blood forming the NT-proXNP analyte. They can

be divided into two main groups: one derived from NT-proBNP and the other from NT-proANP. The NT-proBNP components consisted of several smaller peptides of various lengths, whereas NT-proANP appeared to be more homogeneous in nature. This is consistent with our previous results with the NT-proANP and NT-proBNP assays (13). With the selected antisera, the new assay can detect the majority of the NT-proANP and NT-proBNP forms despite the extensive fragmentation of some of the forms, particularly NT-proBNP (13). There appears to be very little, if any, immunoreactive NT-proXNP in human blood corresponding to proANP and proBNP. The NT-proANP and NT-proBNP antisera used in the current assay cross-react 100% with proANP purified from human atria and with recombinant human proBNP; they therefore should be able to detect any circulating full-length prohormones. The result is consistent with our previous studies with NT-proANP and NT-proBNP (13). Thus, the novel NT-proXNP assay recognizes the same material in blood as individual NT-proANP and NT-proBNP assays.

We tested the performance of the NT-proXNP assay with samples from 100 healthy persons and 478 patients with VHD or CAD. The reference concentration in women was significantly higher than in men. Similar gender differences have been reported previously for BNP and NT-proBNP (16, 17) as well as for ANP and NT-proANP (18, 19). Although the concentrations tended to be slightly higher in women, we did not find any significant gender difference with NT-proANP or NT-proBNP in the present or the previous study (13). We do not know the reason for this discrepancy, but it could be related to the use of assays with various epitope specificities in different studies.

The patients with VHD presumably had more pronounced pressure overload in the heart, and the NT-proXNP, NT-proANP, and NT-proBNP concentrations were significantly higher in those patients than in patients with CAD. This is consistent with previous reports in which ANP concentrations were higher in VHD patients than in CAD patients (20) and in which NT-proANP concentrations were only slightly increased in CAD patients (21). Acute coronary syndromes cause only moderate increases in BNP and NT-proBNP concentrations compared with the increases seen in, e.g., heart failure (22). According to ROC analyses, NT-proXNP seemed to be more clinically efficient than NT-proANP and NT-proBNP in detecting VHD and CAD. The AUC for CAD patients in particular was substantially larger than the AUC for NT-proANP, NT-proBNP, or the arithmetic sum of these two peptides.

BNP and NT-proBNP are often considered to be more useful markers of cardiac dysfunction, particularly impaired left ventricular function, than ANP and NT-proANP (1, 4, 23, 24). In the present study, the AUC for NT-proANP in detecting CAD was, however, larger than the AUC for NT-proBNP ( $P < 0.001$ ). We do not have an

explanation for the discrepancy. Our assays for NT-proANP and NT-proBNP are technically very comparable with each other; they both use full-length recombinant calibrators, and they recognize the majority of the circulating fragments of NT-proANP and NT-proBNP (13). On the other hand, our novel NT-proXNP method has the potential to combine the advantages of both N-terminal peptides.

In conclusion, we present here a novel NT-proXNP immunoassay that can combine the diagnostic information obtainable from the cardiac ANP and BNP systems. In this preliminary study, the assay appears to have higher clinical efficiency than individual assays for NT-proANP or NT-proBNP.

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