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# Alternate Circulating Pro-B-Type Natriuretic Peptide and B-Type Natriuretic Peptide Forms in the General Population

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**Objectives** This study was designed to determine whether alternate pro-B-type natriuretic peptide (proBNP) and BNP forms circulate in the general population.

**Background** Bioactive $BNP_{1-32}$ and NT-$proBNP_{1-76}$ are derived from a precursor molecule, $proBNP_{1-108}$. Recent data suggest that aminodipeptidase-processed forms of $BNP_{1-32}$ ($BNP_{3-32}$) and of $proBNP_{1-108}$ itself ($proBNP_{3-108}$) may circulate and have additional diagnostic potential.

**Methods** Residents (age ≥45 years) of Olmsted County, Minnesota, underwent medical review, echocardiography, and phlebotomy for 2 novel assays specific for $proBNP_{3-108}$ and $BNP_{3-32}$ and 2 commercial assays (Triage BNP and Roche NT-proBNP). Groups included normal subjects (n = 613), cardiovascular disease with normal ventricular function (n = 1,043), preclinical ventricular dysfunction (ALVD, n = 130), and chronic heart failure (HF, n = 52).

**Results** $ProBNP_{3-108}$ levels were above assay detection limits in 68% of normal subjects (50th; 25th to 75th percentiles: 7.85; 3.00 to 22.45 pmol/l) and correlated with age, gender, body size, and renal function and with results of commercial assays. $ProBNP_{3-108}$ levels were higher in ALVD (17.88; 6.07 to 42.76 pmol/l) or HF (42.75; 20.51 to 65.73 pmol/l), where they correlated more strongly with commercial assays. $BNP_{3-32}$ was above assay detection limits in 22% of normal subjects; levels were not correlated with age, body size, or renal function but were higher in HF. Neither novel assay was superior to commercial assays for the detection of ALVD or HF.

**Conclusions** The presence of alternate circulating proBNP and BNP forms provides evidence for diverse proBNP and BNP processing in the general population. The physiologic consequences of these observations, both in terms of assay performance and endogenous BNP bioactivity, deserve further study. (J Am Coll Cardiol 2007;49:1193–202)


Measurement of plasma levels of B-type natriuretic peptide (BNP) aids in the diagnosis of heart failure (HF) (1). However, levels of BNP measured using commercially available assays have shown marked variation even in homogenous populations (1,2). This variability has remained unexplained, as has the seemingly paradoxical sensitive and dose-related vasodilator response to exogenous intravenous administration of BNP in HF patients whose endogenous BNP levels as measured by commercial BNP assays are already extremely high (1–3).

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B-type natriuretic peptide is produced in cardiomyocytes, where it is derived from the 108 amino acid (aa) precursor proBNP. ProBNP is believed to be cleaved by the endoprotease corin upon secretion resulting in the formation of the bioactive 32 aa BNP peptide (referred to as $BNP_{1-32}$ or as $proBNP_{77-108}$) and the inert 76 aa N-terminal peptide (NT)-proBNP ($proBNP_{1-76}$) (Fig. 1). This simple proBNP processing scheme suggests only 2 circulating proBNP-derived peptides, which commercially available BNP or NT-proBNP assays are assumed to specifically detect (Fig. 2, Table 1). Challenging this concept, a recent study of advanced HF patients with high BNP levels, as indicated by a commercial assay, demonstrated an absence of endogenous circulating $BNP_{1-32}$ when a highly accurate mass spectrometry technique was used (4). This led the authors to speculate that alternate forms of proBNP-derived products were responsible for the high levels detected by the commercial assay. Although this study did not identify what

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**Abbreviations and Acronyms**

**aa** = amino acid
**ALVD** = asymptomatic left ventricular dysfunction
**BNP** = B-type natriuretic peptide
**BSA** = body surface area
**CV** = cardiovascular
**EF** = ejection fraction
**GFR** = glomerular filtration rate
**HF** = heart failure
**MDC** = minimal detectable concentration
**NT** = N-terminal
**ROC** = receiver operating characteristic

these altered forms might be, others have suggested that intact proBNP$_{1\text{-}108}$ may be one such form (5–8) and that commercial BNP and NT-proBNP assays potentially cross-react with proBNP$_{1\text{-}108}$ (9). Further, BNP$_{1\text{-}32}$ has been shown to undergo further degradation both in vitro (8) and in vivo (7,10), generating a BNP form that lacks the two N-terminal aa residues (BNP$_{3\text{-}32}$ or proBNP$_{79\text{-}108}$). Very recently, Brandt et al. (11) demonstrated that this degradation occurred by the action of purified dipeptidyl peptidase IV in vitro. This aminodipeptidase preferentially cleaves dipeptides from peptides with a proline or alanine in the second N-terminal position. Importantly, proBNP also possesses a proline in the second position. Thus, it too may be susceptible to dipeptidyl peptidase IV (Figs. 2 and 3) and be processed to proBNP$_{3\text{-}108}$, but this has not been examined.

Recognizing the growing evidence for diverse circulating proBNP products, our objectives were to determine whether 2 likely alternate proBNP and BNP forms (BNP$_{3\text{-}32}$ and proBNP$_{3\text{-}108}$) circulate in human plasma in the general population. We also sought to determine if levels of these altered forms differ according to age, gender, body size, renal function, or the presence of CV disease, ALVD, or chronic HF within the general population. Lastly, we sought to compare the ability of these novel assays to identify persons with ALVD or chronic HF with that of commercial assays, specifically the Biosite Triage BNP assay and the Roche NT-proBNP assay.

## Methods

This study was approved by the Mayo Institutional Review Board.

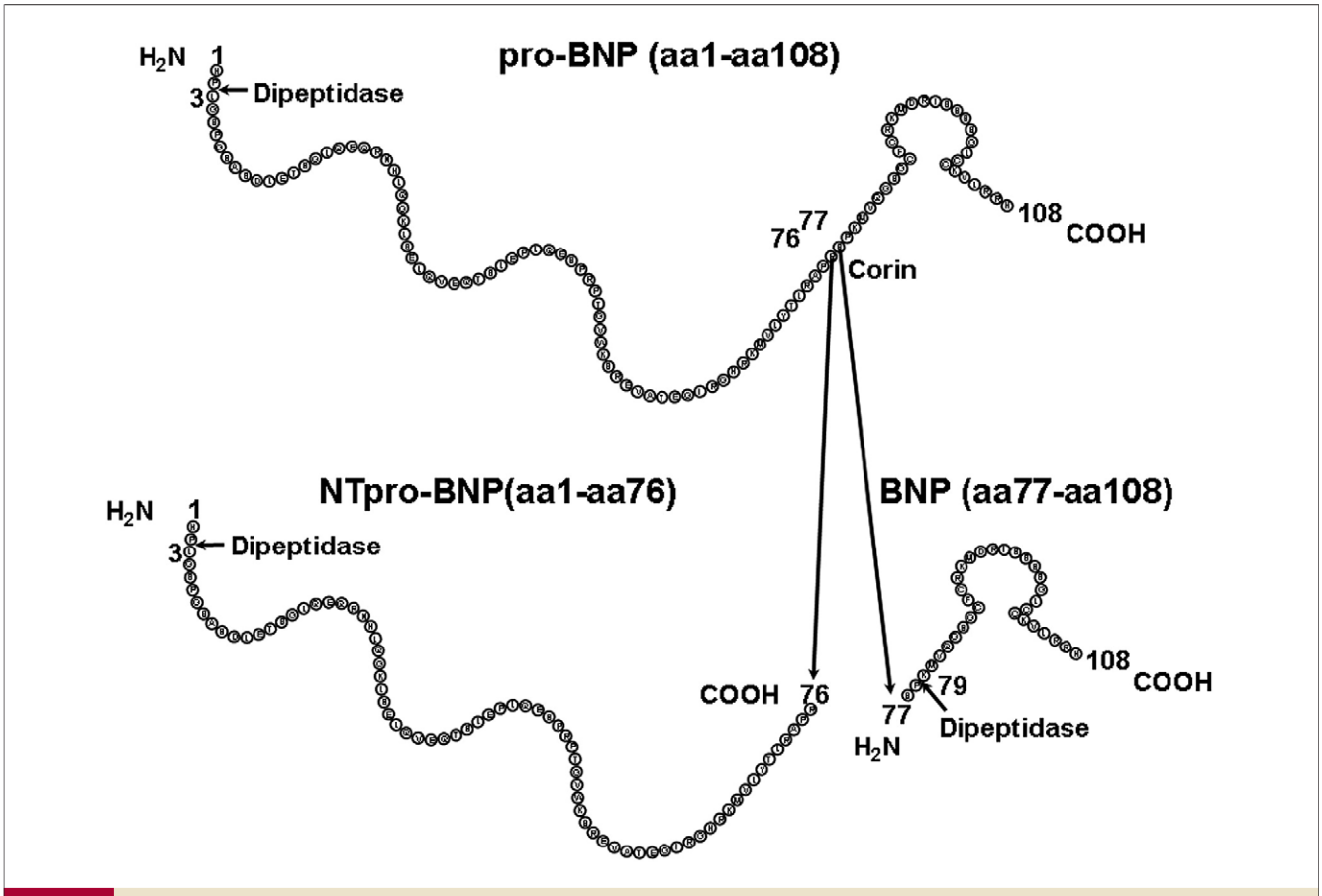


**Figure 1** ProBNP Processing

ProBNP is a 108-amino acid (aa) precursor protein which is cleaved by the endoprotease corin to form the bioactive 32 aa BNP peptide (referred to as BNP$_{1\text{-}32}$ or as proBNP$_{77\text{-}108}$) and the inert 76 aa N-terminal-proBNP peptide (proBNP$_{1\text{-}76}$). Each fragment undergoes further N-terminal dipeptidase digestion, as might proBNP itself. BNP = B-type natriuretic peptide.

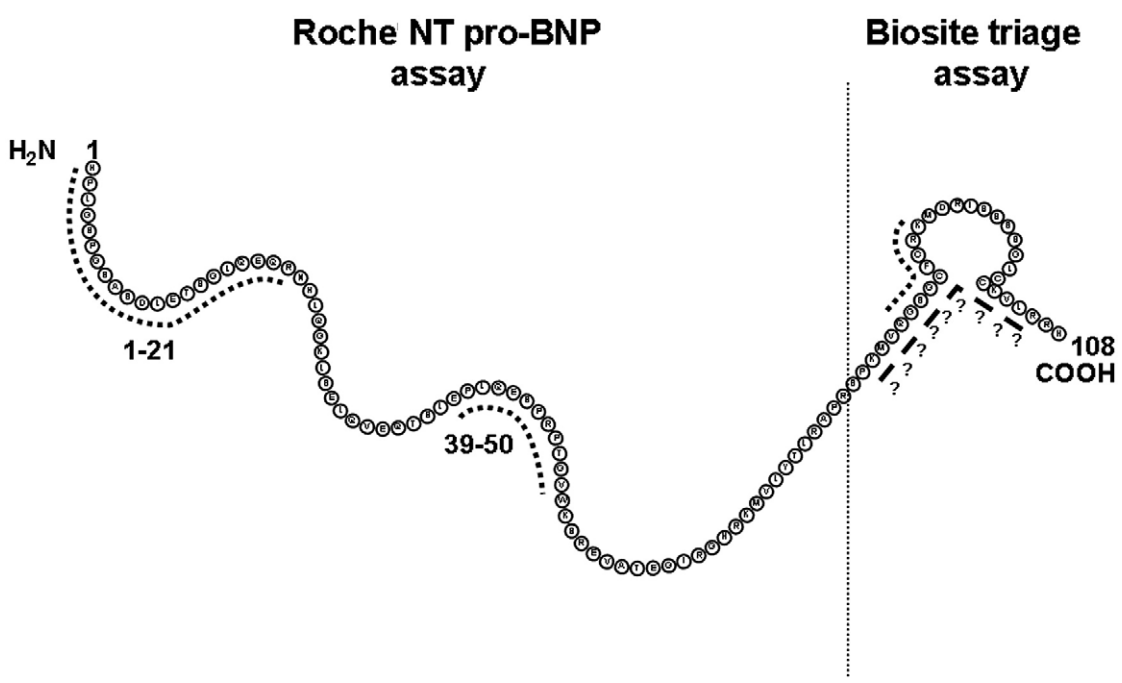


**Figure 2** Commercial BNP or NT-proBNP Assays

Commercial BNP or NT-proBNP assays utilize dual antibody systems which recognize 2 separate segments of $BNP_{1-32}$ or of NT-$proBNP_{1-76}$, respectively. **(Left)** The Roche NT-proBNP assay combines a capture antibody targeting proBNP aa 1-21 with a detection antibody recognizing proBNP aa 39-50. **(Right)** The Biosite Triage assay combines the monoclonal Scios 106.3 antibody, which binds somewhere in the proBNP aa 82-90 region, with the Biosite polyclonal antibody, which binds somewhere in the proBNP aa 79-108 region. Other abbreviations as in Figure 1.

**Study setting, population sampling, subject recruitment, and characterization.** The characteristics of the Olmsted County, Minnesota, population, unique aspects of community-based research, and methods used to sample, recruit, and characterize the population have been previously described (12). Briefly, a total of 2,042 subjects were recruited from a random sample of the population >44 years. Each subject's medical record was reviewed by trained nurse chart abstractors using established criteria for hypertension, myocardial infarction, or HF (12). Clinical diagnoses of coronary artery disease, valvular heart disease, cardiomyopathy, atrial fibrillation, transient ischemic attack or stroke, and diabetes mellitus were also recorded.

**Doppler echocardiography.** Echocardiographic methods for the measurement of EF and diastolic function in this study population have been extensively described (12). Diastolic function was categorized as normal, mild dysfunction ("impaired relaxation"), moderate dysfunction ("pseudonormal filling"), and severe dysfunction ("restrictive filling") (12). Of 2,042 subjects, 1,838 (90%) had EF assessment, determination of diastolic function, and ascertainment of the presence or absence of CV disease, allowing classification into the subject groups.

**Subject groups.** Subjects were classified into 4 groups (Table 2): 1) normal control subjects with systolic blood pressure <140 mm Hg at the time of echocardiography, body mass index <30 $kg/m^2$, normal systolic function (EF >40%) and normal or only mildly abnormal diastolic function in the absence of CV disease (normal group); 2) subjects with CV disease but normal systolic function (EF >40%) and normal or only mildly abnormal diastolic function (CV disease group); 3) subjects with ALVD, defined as EF ≤40% and/or moderate to severe diastolic dysfunction but no HF diagnosis (ALVD group); and 4) subjects with chronic HF, defined as those diagnosed with HF before entry into the study (HF group).

**Novel immunoassay development.** Two potential alternate circulating proBNP products were identified on the basis of previous studies. Shimizu et al. (7) reported that 94% of spiked recombinant $BNP_{1-32}$ underwent proteolytic cleavage of its N-terminal Ser-Pro aa residues when incubated in whole blood, generating the dominant fragment $BNP_{3-32}$. This form of N-terminal X-Pro trimming has been observed with other signal peptides (13) and demonstrated to occur in serum (14) and intracellular vesicles (15). Trimming by aminodipeptidases such as dipeptidyl peptidase IV has been suggested to explain the observed proteolytic cleavage of recombinant $BNP_{1-32}$ in vitro, with similar occurrence in vivo (10). Aminodipeptidase cleavage could likewise occur at the N-terminal His-Pro motif of $proBNP_{1-108}$ were $proBNP_{1-108}$ to circulate. Indeed, preliminary experiments using a chip-based platform coated

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**Table 1 Assay Details**

| | Capture Antibody | Detection Antibody | Calibrating Peptide | MDC (pmol/l) | Intra-CV (%) | Inter-CV (%) | Total CV (%) | Cross-Reactivity | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | $BNP_{1-32}$ | $BNP_{3-32}$ | $proBNP_{3-108}$ | $proBNP_{1-76}$ | $proBNP_{1-108}$ |
| Biosite $proBNP_{3-108}$ | $proBNP_{3-108}$ N-terminal aa 3 | Biosite polyclonal* | $proBNP_{3-108}$ | 3.00 | 24 | 13 | — | No | No | NA | No | No |
| Biosite $BNP_{3-32}$ | $BNP_{3-32}$ N-terminal aa 3 | Biosite polyclonal* | $BNP_{3-32}$ | 3.05 | 23 | 7 | — | No | NA | No | No | No |
| Biosite Triage BNP | Scios 106.3 monoclonal† | Biosite polyclonal* | $BNP_{1-32}$ | 2.02 | 8.8–11.6 | — | 9.9–12.2 | NA | Yes | ? | No | ? |
| Roche NT-proBNP | proBNP aa 1-21 | proBNP aa 39-50 | $proBNP_{1-76}$ | 0.59 | 1.8–2.7 | — | 2.2–3.2 | No | No | ? | NA | ? |

*Biosite polyclonal antibody binds somewhere in the 79–108 region of $proBNP_{1-108}$ ($BNP_{3-32}$); †Scios 106.3 monoclonal antibody binds somewhere in the 82–90 region of $proBNP_{1-108}$.
CV = assay coefficient of variation; MDC = the minimal concentration detected by the assay based on the calibrating peptide for each assay system in pmol/l; NA = not applicable; NT = N-terminal; proBNP = proB-type natriuretic peptide.

with anti-BNP antibodies and mass analysis have demonstrated the existence of both $proBNP_{3-108}$ and $BNP_{3-32}$ in isolated patient plasma samples (16). On the basis of these data, two novel immunoassays were developed by Biosite; the Biosite $proBNP_{3-108}$ assay and the Biosite $BNP_{3-32}$ assay (described hereafter and in Fig. 3). Proteolysis of synthetic BNP was subsequently confirmed at the Biosite laboratory, where recombinant $BNP_{1-32}$ (nesiritide) or expressed $proBNP_{1-108}$ was spiked into ethylenediaminetetraacetic acid (EDTA) anti-coagulated plasma and analyzed after incubation using the $BNP_{3-32}$ and $proBNP_{3-108}$ assays. The half-time for the formation of $BNP_{3-32}$ from $BNP_{1-32}$ was <30 min, whereas that of $proBNP_{3-108}$ from $proBNP_{1-108}$ was approximately 2 h.

**$proBNP_{3-108}$ assay.** A monoclonal antibody specific to the N-terminal end of $proBNP_{3-108}$ was raised by immunizing mice with a $proBNP_{3-108}$ peptide segment synthesized from the $proBNP_{3-108}$ sequence. Antibody selection by phage panning ensured specificity for the N-terminal end of $proBNP_{3-108}$. This antibody was paired with the Biosite polyclonal antibody used in the Triage assay, which binds somewhere in the $proBNP_{79-108}$ region, to produce the $proBNP_{3-108}$ assay (Fig. 3, Table 1). To confirm specificity of the assay for the N-terminus of $proBNP_{3-108}$, $proBNP_{1-108}$ was cloned and expressed (mammalian cells). The measured cross-reactivity with $proBNP_{1-108}$ was <10%. $proBNP_{3-108}$ was cloned and expressed (mammalian cells) and used to calibrate and define the MDC of the $proBNP_{3-108}$ assay.

**$BNP_{3-32}$ ($proBNP_{79-108}$) assay.** A monoclonal antibody specific to the N-terminal end of $BNP_{3-32}$ was raised by immunizing mice with a $BNP_{3-32}$ peptide segment synthesized from the $BNP_{3-32}$ sequence. Antibody selection by phage panning ensured specificity for the N-terminal end of $BNP_{3-32}$. This antibody was paired with the Biosite polyclonal antibody used in the Triage assay to produce the $BNP_{3-32}$ assay (Fig. 3, Table 1). To confirm specificity, cross-reactivity tests with expressed $proBNP_{1-108}$, $proBNP_{3-108}$ and $BNP_{1-32}$ were performed. Cross-reactivity was <10% in all cases. The Triage BNP test was shown to exhibit 100% cross-reactivity with $BNP_{3-32}$ by both spike and recovery experiments with expressed $BNP_{3-32}$ and by lack of a signal change during proteolysis of $BNP_{1-32}$ to $BNP_{3-32}$ in EDTA plasma. $BNP_{3-32}$ was used to calibrate the $BNP_{3-32}$ assay and to define the MDC.

**Plasma collection and analysis.** Blood was collected from each subject in the fasting state in EDTA-treated tubes and placed on ice. After centrifugation at 2,500 rpm and 3°C, the plasma was stored at −80°C. A 1-ml aliquot of frozen plasma was shipped to Biosite Diagnostics (San Diego, California), where nonextracted samples were batch analyzed using the $proBNP_{3-108}$, $BNP_{3-32}$ and Triage immunoassays. A second aliquot was analyzed at the Mayo Clinic Core Laboratory (Rochester, Minnesota) using the Roche NT-proBNP assay as previously described (17). Assay performance characteristics are summarized in Table 1 and

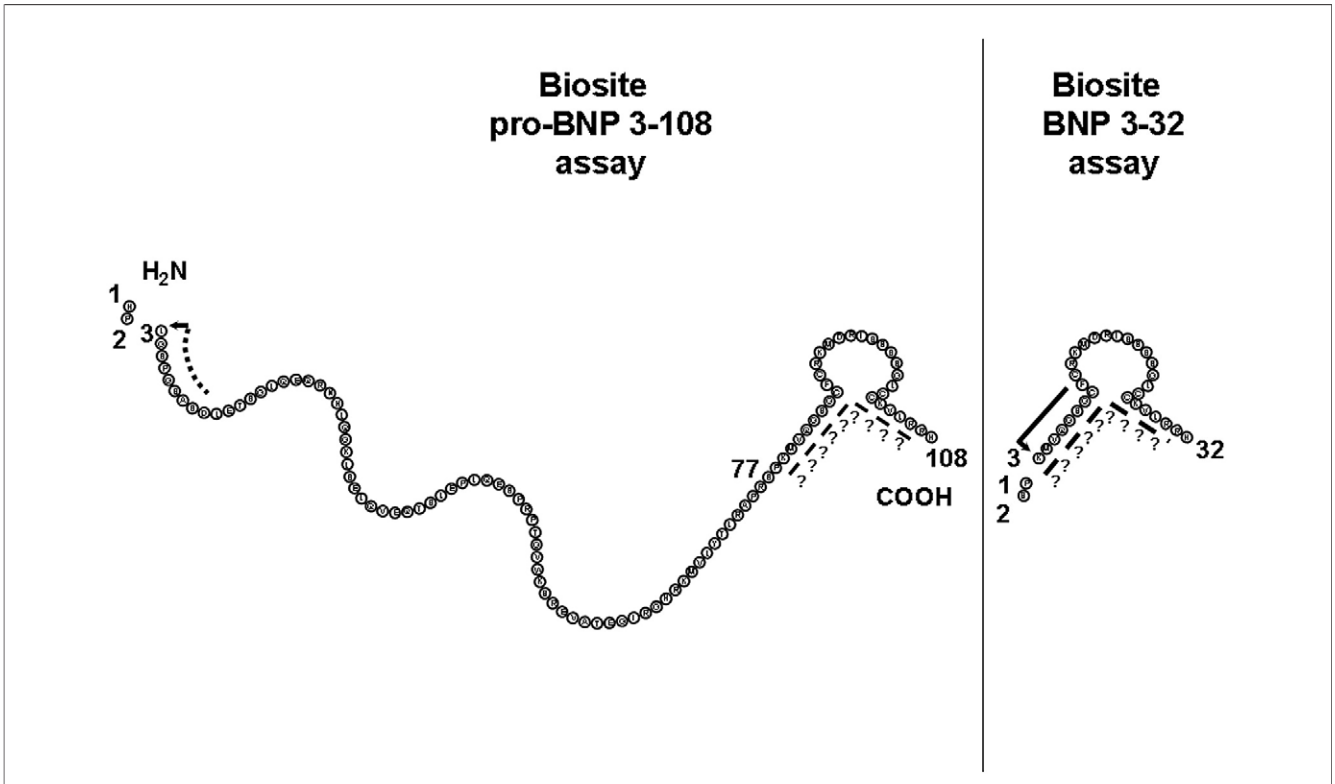


**Figure 3** **Biosite ProBNP$_{3\text{-}108}$ and BNP$_{3\text{-}32}$ Assays**

**(Left)** The proBNP$_{3\text{-}108}$ assay combines an antibody specific for the N-terminus aa 3 of proBNP with the Biosite polyclonal antibody used in the Triage assay. **(Right)** The BNP$_{3\text{-}32}$ assay combines an antibody specific for the N-terminus aa 3 of BNP with the Biosite polyclonal antibody used in the Triage assay. Abbreviations as in Figure 1.

illustrated in Figures 2 and 3. Serum creatinine was measured in each subject at the Mayo Central Clinical Laboratory using the standard colorimetric Jaffe reaction. Glomerular filtration rate (GFR) was estimated using the modification of diet in renal disease equation.

**Statistical methods.** Based on the skewed distribution of assay results, natural log transformation was used in regression analyses to satisfy modeling assumptions. Comparisons between groups used ANOVA for continuous variables (with log transformation as appropriate) and chi-square

**Table 2** **Patient Characteristics**

| | Normal (n = 613) | CV Disease (n = 1,043) | ALVD (n = 130) | Chronic HF (n = 52) | p Value* |
|---|---|---|---|---|---|
| Age (range) (yrs) | 57 (45–96) | 63 (45–94) | 70 (47–89) | 73 (48–89) | <0.01 |
| Male (%) | 46 | 48 | 47 | 64 | 0.1 |
| BSA ($m^2$) | 1.83 ± 0.20 | 1.94 ± 0.24 | 1.86 ± 0.23 | 1.90 ± 0.26 | <0.01 |
| Creatinine (mg/dl) | 1.02 ± 0.15 | 1.07 ± 0.22 | 1.09 ± 0.21 | 1.25 ± 0.45 | <0.01 |
| GFR (ml/min/1.73 $m^2$) | 76.4 ± 17.3 | 82.7 ± 33.6 | 66.7 ± 34.7 | 63.9 ± 35.7 | <0.01 |
| EF (%) | 63 ± 5 | 64 ± 6 | 59 ± 11 | 47 ± 14 | <0.01 |
| Diastolic dysfunction (%) | | | | | <0.01 |
| Normal | 92 | 71 | 2 | 4 | |
| Mild | 8 | 29 | 4 | 37 | |
| Moderate | 0 | 0 | 89 | 37 | |
| Severe | 0 | 0 | 6 | 22 | |
| Hypertension (%) | 0 | 54 | 60 | 81 | <0.01 |
| Coronary disease (%) | 0 | 14 | 29 | 85 | <0.01 |
| Obesity (%) | 0 | 50 | 29 | 40 | <0.01 |
| Diabetes (%) | 0 | 10 | 9 | 25 | <0.01 |

Data are mean ± SD except where indicated. *Chi-square or analysis of variance p values as appropriate.

ALVD = asymptomatic left ventricular dysfunction; BSA = body surface area; CV = cardiovascular; EF = ejection fraction; GFR = glomerular filtration rate; HF = heart failure.

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analysis for discrete variables. Unadjusted and adjusted associations of assay results were evaluated using Spearman's correlation coefficient and linear least-squares regression, respectively. The ability of each assay to detect ALVD or HF was evaluated using receiver operating characteristic (ROC) curves. The area under each ROC curve was used to test the null hypothesis of true area = 0.5. Statistical significance was judged at the 0.05 level.

## Results

**Subject groups.** Subject group characteristics are shown in Table 2.

**Assays results in persons without CV disease or ventricular dysfunction (normal group).** $ProBNP_{3\text{-}108}$ levels were above the MDC in 68% of normal subjects. Levels of $proBNP_{3\text{-}108}$ increased with age ($R = 0.12$, $p = 0.009$), were higher in women than in men ($20.52 \pm 28.88$ pmol/l vs. $12.81 \pm 17.09$ pmol/l, $p < 0.0001$), and decreased with increasing BSA ($R = -0.13$, $p = 0.005$) or increasing GFR ($R = -0.20$, $p < 0.001$). Persons with detectable $proBNP_{3\text{-}108}$ were older ($p = 0.001$), more frequently female ($p < 0.001$), and had lower GFR ($p < 0.001$) and BSA ($p = 0.014$) than those with results below the MDC. In multivariate linear regression modeling adjusting for age, gender, creatinine, and BSA, the positive association between log $proBNP_{3\text{-}108}$ and age, female gender, and creatinine remained significant (adjusted $p < 0.05$ for all).

B-type $natriuretic_{3\text{-}32}$ levels were above the MDC in only 22% of normal subjects. There was no association between $BNP_{3\text{-}32}$ levels and age, BSA, or GFR ($p > 0.05$ for all) and borderline significant association with gender ($p = 0.038$). Persons with detectable $BNP_{3\text{-}32}$ levels did not differ from those with levels below the MDC in regards to age, gender, body size, or renal function.

The Biosite Triage and Roche NT-proBNP results were above the MDC in 77% and 94%, respectively, of normal subjects. Both commercial assay results were positively associated with age, female gender, worsening renal function, and smaller body size, whereas persons with detectable results were older, more likely female, and had lower GFR and BSA than persons with results below the MDC (data not shown).

**Assay results in persons with CV disease, ALVD, or chronic HF.** The percentage of persons with assay results above the MDC increased across the four groups for the $proBNP_{3\text{-}108}$, Biosite Triage, and Roche NT-proBNP assays, but not the $BNP_{3\text{-}32}$ assay (Fig. 4). Assay results are summarized in Figure 5, where immunoreactivity in pmol/l was calculated for each assay using the molecular weight of its specific calibrating peptide. $proBNP_{3\text{-}108}$ (ANOVA $p < 0.001$), $BNP_{3\text{-}32}$ (ANOVA $p = 0.013$), Biosite Triage (ANOVA $p < 0.001$), and Roche NT-proBNP (ANOVA $p < 0.001$) levels varied significantly across the groups. Adjusting for age, gender, creatinine, and BSA, $proBNP_{3\text{-}108}$ levels were higher in ALVD than CV disease and higher in HF than ALVD. B-type $natriuretic\ peptide_{3\text{-}32}$ levels were higher in HF than ALVD. Both the Biosite Triage and the Roche NT-proBNP assay results were increased in each group as compared with the previous one. Based on median molar immunoreactivity, the ratio of $proBNP_{3\text{-}108}$, Biosite Triage and the Roche NT-proBNP was essentially 1:1:1 except in chronic HF, where higher immunoreactivity was observed for the Roche NT-proBNP assay.

Receiver operating characteristic analyses for detection of ALVD and HF are shown in Figure 6. Although both $proBNP_{3\text{-}108}$ and $BNP_{3\text{-}32}$ demonstrated some diagnostic utility, neither was superior to the Biosite Triage or Roche NT-proBNP assays for the diagnosis of ALVD or HF.

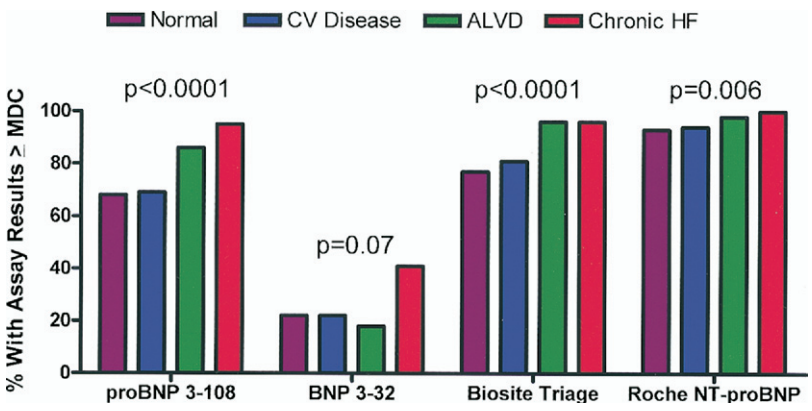


**Figure 4** Percentage of Subjects With Detectable Assay Results

**Bars** represent the percentage of subjects in each group (see text) with measured levels above the minimal detectable concentration of each assay. Chi-square p values for between-group comparisons are given for each assay. ALVD = asymptomatic left ventricular dysfunction; BNP = B-type natriuretic peptide; CV = cardiovascular; HF = heart failure; MDC = minimal detectable concentration; NT = N-terminal.

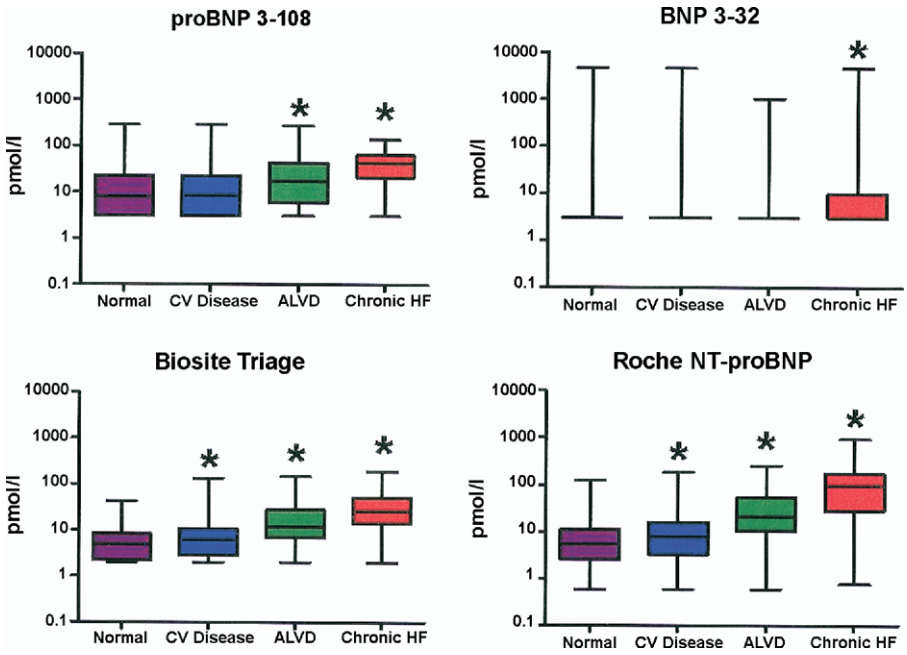


**Figure 5** **Assay Results by Subject Group**

Boxplots showing median, interquartile range, minimum, and maximum observed immunoreactivity in pmol/l by subject group (see text). For each assay, *represents $p < 0.05$ for comparison of mean values between that group and the previous group, adjusting for age, gender, creatinine, and body surface area. Abbreviations as in Figure 4.

In persons with normal systolic and normal or only slightly abnormal diastolic function (normal and CV disease groups combined), there was moderate correlation between the $proBNP_{3\text{-}108}$ and Biosite Triage results ($R = 0.32$, $p < 0.001$) as well as between $proBNP_{3\text{-}108}$ and Roche NT-proBNP results ($R = 0.34$, $p < 0.001$). The correlation between $proBNP_{3\text{-}108}$ and Biosite Triage results ($R = 0.65$, $p < 0.001$) and between $proBNP_{3\text{-}108}$ and Roche NT-proBNP results ($R = 0.61$, $p < 0.001$) appeared stronger in persons with ventricular dysfunction with or without HF (ALVD and HF groups combined). The $BNP_{3\text{-}32}$ assay results did not correlate with the Biosite Triage or the Roche NT-proBNP assay results in either of these groups.

## Discussion

This study, using both novel and commercially available proBNP product assay systems, suggests that proBNP processing is diverse, as $proBNP_{3\text{-}108}$ was found to circulate in subjects from the general population, even in those without CV disease or ventricular dysfunction. Levels of $proBNP_{3\text{-}108}$ correlated with age, gender, body size, and renal function and with the results of commercially available proBNP product assays. $proBNP_{3\text{-}108}$ levels were higher in those with ALVD or chronic HF, where they were more strongly correlated with results from the Biosite Triage and Roche NT-proBNP assays. B-type natriuretic $peptide_{3\text{-}32}$ was less frequently detected and, when present, was not correlated with age, body size, or renal function, although levels of $BNP_{3\text{-}32}$ were higher in subjects with chronic HF. Neither the $proBNP_{3\text{-}108}$ nor the $BNP_{3\text{-}32}$ assay was superior to commercially available assays for the detection of ALVD or HF.

**$proBNP_{3\text{-}108}$.** Previous studies using high-pressure liquid chromatography have suggested that intact proBNP circulates in HF (5–8). However, these studies involved very small numbers of patients with severe HF; used small, selected groups of normal individuals as control subjects; and could not discriminate between $proBNP_{1\text{-}108}$ and $proBNP_{3\text{-}108}$. Here selection bias was reduced by using a population-based cohort. The current data further extend previous high-pressure liquid chromatography studies by demonstrating that the aminodipeptidase proteolyzed product of $proBNP_{1\text{-}108}$ ($proBNP_{3\text{-}108}$) circulates, even in persons without CV disease or ventricular dysfunction.

The current findings are also consistent with a previous attempt at the development of a $proBNP_{1\text{-}108}$-specific assay that combined a monoclonal antibody targeting proBNP aa 75-80 with a polyclonal antibody directed against a site on $BNP_{1\text{-}32}$ ($proBNP_{77\text{-}108}$) (18). Our dual antibody system, however, spans the full polypeptide sequence of $proBNP_{3\text{-}108}$ more completely than the previous assay. This should avoid cross-reaction with other potential fragments formed by endoprotease digestion along the N-terminal tail

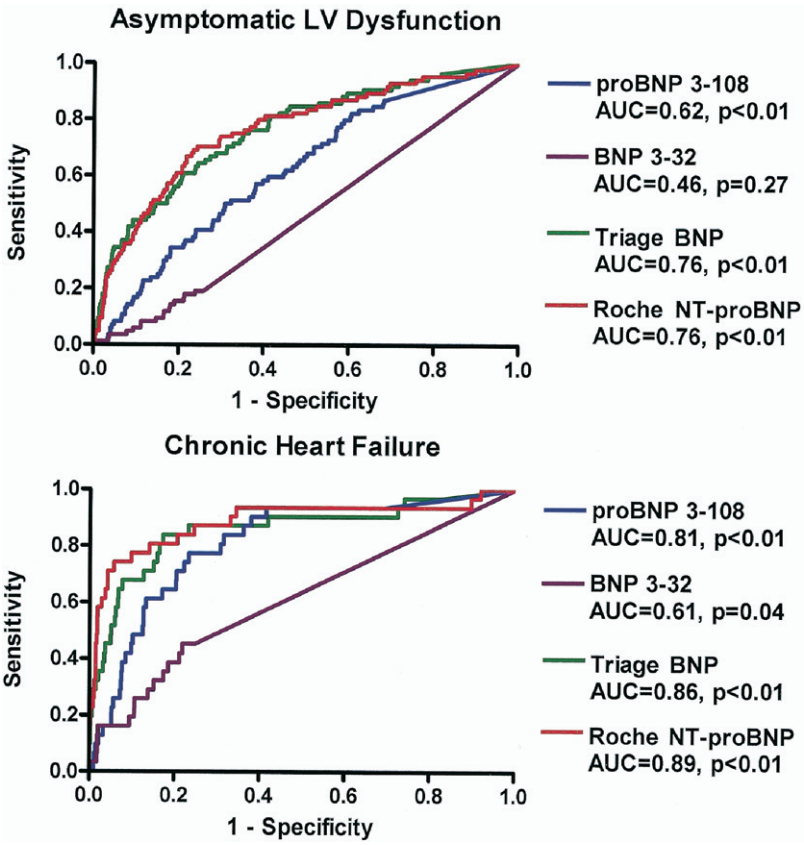


**Figure 6** ROC Curves for Detection of ALVD and HF

**(A)** ROC curves for detection of ALVD using the different assays. **(B)** ROC curves for detection of HF using the different assays. AUC = area under the curve; other abbreviations as in Figure 4.

of proBNP. When our findings are taken together with previous studies, one must conclude that $proBNP_{1\text{-}108}$ is released into the circulation even in normal human subjects in the general population. $ProBNP_{1\text{-}108}$ then undergoes processing to $ProBNP_{3\text{-}108}$, $ProBNP_{1\text{-}76}$ (NT-proBNP) and $BNP_{1\text{-}32}$, the latter further degraded to $BNP_{3\text{-}32}$ as discussed below. Further studies are clearly needed to understand the biologic actions of these processed forms.

The association of $proBNP_{3\text{-}108}$ with age, gender, body size, and renal function in the normal cohort of this population-based study deserves attention. We and others have previously reported that commercially available assay results show similar associations. Alterations in BNP or NT-proBNP synthesis, degradation or clearance with age, gender, body size, and renal function have been postulated to explain the variation of commercial assay levels with these variables (17,19,20). However, as commercially available assays may cross-react with $proBNP_{1\text{-}108}$ and $proBNP_{3\text{-}108}$ (9), altered proBNP processing with increases in proBNP or its aminodipeptidase proteolyzed fragment may also contribute to the variability of commercial assays with age, gender, renal function, and body size.

More importantly, the markedly but variably increased results on commercial assays in persons with HF may be due in part to altered proBNP processing with elevation of circulating $proBNP_{3\text{-}108}$ (and potentially intact $proBNP_{1\text{-}108}$). A preliminary report from Dries et al. (21) described an inverse relationship between corin and BNP messenger ribonucleic acid in cardiac tissue harvested from patients with end-stage HF, suggesting that the capacity to process proBNP may decrease as the signal for enhanced proBNP production increases. The increased levels of $proBNP_{3\text{-}108}$ in ALVD and HF, nearly equivalent relative molar concentrations of $proBNP_{3\text{-}108}$, Biosite Triage and Roche NT-proBNP in ALVD, and enhanced correlation between $proBNP_{3\text{-}108}$ and the Biosite Triage and Roche NT-proBNP assay results in persons with LV dysfunction are consistent with this concept. Further, the higher median molar immunoreactivity observed with the Roche NT-proBNP assay relative to the other assays in chronic HF may be due to more complete cross-reaction with all $proBNP_{3\text{-}108}$, NT-$proBNP_{1\text{-}76}$ and other fragments, rather than to reduced clearance of the NT- $proBNP_{1\text{-}76}$ fragment as previously postulated.

**$BNP_{3-32}$.** In this population-based study, levels of $BNP_{3-32}$ were above the MDC in only a small percentage of normal subjects and in less than half of those with HF. Further, despite the previously mentioned 100% cross-reactivity of the Biosite Triage assay with $BNP_{3-32}$ demonstrated during assay development, levels of $BNP_{3-32}$ did not correlate with the results of the Biosite Triage assay. These findings may be interpreted in a number of ways. One could conclude that little $BNP_{1-32}$ is subject to aminopeptidase processing in vivo or that $BNP_{3-32}$ exists only transiently and is subsequently further digested. However, unlike ANP, both $BNP_{1-32}$ and $BNP_{3-32}$ were resistant to degradation by purified neutral endopeptidases in vitro (11). Further, the in vitro data in this study suggest that $BNP_{1-32}$ is rapidly and stably digested to $BNP_{3-32}$. Alternatively, one could also conclude that the low levels of $BNP_{3-32}$ reflect a low level of proBNP cleavage by corin, a conclusion supported by the lack of correlation between the Biosite Triage assay and the $BNP_{3-32}$ assay, the observation that $BNP_{3-108}$ is reliably detected at significant levels, and the observed correlation between $BNP_{3-108}$ and the Biosite Triage assay. These data support the emerging concept that commercial assays targeting $BNP_{1-32}$ are not specific for $BNP_{1-32}$ and may be cross-reacting with proBNP, its aminodipeptidase proteolyzed fragment, or other alternatively processed forms.

**HF as a natriuretic peptide-deficient state.** Historically, HF was first postulated to be a natriuretic peptide-deficient state, as immunohistochemistry studies and bioactivity assays indicated that atria from hamsters in HF were deficient in atrial natriuretic peptide (22,23). However, development of plasma natriuretic peptide assays subsequently indicated increased circulating natriuretic peptide levels in HF (24). Yet instead of increased natriuretic effect expected with increased levels, overt HF patients display fluid and salt retention. Mechanisms for end-organ resistance to elevated natriuretic peptide levels have been invoked to explain this apparent paradox (25,26). However, our data lend further support to the alternative and highly novel hypothesis that abnormal proBNP processing, with reduced levels of biologically active natriuretic peptide forms, may contribute to this paradox. Possible underlying mechanisms for altered proBNP processing include inadequate corin gene expression (21), corin mutations (27), alternate proBNP processing by furin (28), or differential degradation by neutral endopeptidase (29). Preliminary findings from an in vitro study demonstrated that $BNP_{3-32}$ activated the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) to a similar extent as $BNP_{1-32}$ in cultured cardiac fibroblasts, whereas $proBNP_{1-108}$ had no bioactivity (30). Although this in vitro study suggests that $BNP_{3-32}$ is as active as $BNP_{1-32}$, a recent in vivo study showed reduced bioactivity (hypotensive, natriuretic, and cGMP generation effects) with $BNP_{3-32}$ when equimolar doses of $BNP_{3-32}$ and $BNP_{1-32}$ were infused (31). Interestingly, the reduced bioactivity was associated with a similarly reduced increment in BNP immunoreactivity in response to the infusion, questioning the stability of $BNP_{3-32}$ in vivo. Further studies are needed to better understand the role of alternate BNP processing as well as the bioactivity and degradation of alternately processed forms in HF.

**Study limitations.** This study does not account for all possible altered forms of proBNP or BNP and cannot define the extent to which commercial assays cross-react with these alternate forms. Because of the use of expressed $BNP_{3-108}$ to calibrate the assay and the possible effect of glycosylation changes in native compared to expressed protein (32), conclusions could not be made based on exact value assignments (absolute concentration) of the Biosite $proBNP_{3-108}$ assay. As aminodipeptidase processing has been demonstrated to occur in vitro, sample handling may contribute to results of the $BNP_{3-32}$ assay. However, samples in this study were collected and stored in a highly consistent fashion.

## Conclusions

This study provides further evidence that proBNP processing is diverse, even in individuals without CV disease or ventricular dysfunction. Further studies are needed to ascertain the level to which commercial BNP assays cross-react with these and other alternate proBNP products and to define the biologic activity of circulating alternate proBNP products. We speculate that commercial proBNP product assays, proven to be excellent biomarkers reflective of HF presence and severity, may not be good indicators of endogenous natriuretic peptide bioactivity.

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