

# Specificity of B-Type Natriuretic Peptide Assays: Cross-Reactivity with Different BNP, NT-proBNP, and proBNP Peptides

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**BACKGROUND:** B-type natriuretic peptides (BNPs) are used clinically to diagnose and monitor heart failure and are present in the circulation as multiple proBNP-derived fragments. We investigated the specificity of BNP immunoassays with glycosylated and nonglycosylated BNP, N-terminal proBNP (NT-proBNP), and proBNP peptides to probe the cross-reactivity of each assay.

**METHODS:** Nine B-type natriuretic peptides were studied, including synthetic and recombinant BNP (Shionogi, Scios, Mayo), human and synthetic glycosylated and nonglycosylated NT-proBNP (HyTest, Roche Diagnostics), and human glycosylated and nonglycosylated proBNP (HyTest, Scios). Five BNP [Abbott, Abbott POC, Alere, Beckman Coulter, Siemens (Centaur)], 9 NT-proBNP [Ortho-Clinical Diagnostics, Roche, Response, bioMerieux, Siemens (Dimension, Immulite, Stratus CS), Mitsubishi] and 3 research-use-only proBNP immunoassays [Biosite (Alere), Bio-Rad, Goetze] were evaluated. Specificity was assessed by calculating the recovery between baseline and peptide-spiked human plasma pools at target concentrations of 100 ng/L BNP, 300 ng/L proBNP, or 450 ng/L NT-proBNP. All assays were performed in duplicate.

**RESULTS:** BNP and NT-proBNP assays demonstrated substantial cross-reactivity with proBNP peptides. NT-proBNP assays do not detect glycosylated forms of either NT-proBNP or proBNP. proBNP assays preferentially detect the BNP 1–32 peptide and have minimal cross-reactivity with BNP peptides and glycosylated proBNP.

**CONCLUSIONS:** BNP or NT-proBNP results are not transferable among the current existing immunoassays owing to their differences in cross-reactivity and ability to detect various glycosylated forms of proBNP-derived

fragments. Opportunities remain to standardize and harmonize BNP and NT-proBNP assays, as well as to develop specific proBNP assays, to widen their clinical scope of use.

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B-type natriuretic peptide (BNP)<sup>8</sup> and N-terminal proBNP (NT-proBNP) are globally endorsed in clinical guidelines as biomarkers to aid in the diagnosis of heart failure and to monitor disease progression (1–3). BNP is a circulating peptide hormone synthesized and released as a precursor protein in response to increased myocardial wall stress owing to volume or pressure overload and to other conditions such as myocardial ischemia or inflammation. Intracellular processing produces the propeptide proBNP [amino acid (aa) 1–108], which is subsequently cleaved by furin into equimolar amounts of the active peptide BNP (proBNP 77–108 or BNP 1–32) and the biologically inert NT-proBNP (proBNP 1–76 or NT-proBNP 1–76). The bioactive BNP 1–32 is further processed by receptors or enzymes like neprilysin, dipeptidyl peptidase IV, insulin degrading enzyme, meprin, and potentially other enzymatic pathways which may be identified in the future (4–6). Multiple plasma proBNP-derived peptides are present in heart failure patients, of which the truncated BNP 3–32, BNP 4–32, and BNP 5–32 peptides (7–9) and the glycosylated form of NT-proBNP are most prevalent. Very small amounts of intact BNP 1–32 remain in the circulation.

The multiple circulating BNP fragments, along with proBNP and NT-proBNP, collectively form the B-type natriuretic peptide family. Immunoassays for BNP utilize a variety of antibodies, both monoclonal and polyclonal, and diverse calibrator materials. Therefore, there

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<sup>7</sup> Nonstandard abbreviations: BNP, B-type natriuretic peptides; NT-proBNP, N-terminal-proBNP; aa, amino acid; ARNI, angiotensin receptor-neprilysin inhibitors.

are substantial differences in patient specimens between BNP methods, even for assays that use identical antibody configurations on different analytical platforms (10, 11). NT-proBNP assays are generally considered harmonized because all utilize one manufacturer's antibodies and calibrators (Roche Diagnostics) which are then configured to other manufacturers' immunoassay platforms. However, there are still analytical differences between NT-proBNP assays from different manufacturers that can be attributed partially to different specimen types and platforms (12, 13), indicative of a larger need for global standardization of NP assays. Previous studies have demonstrated that proBNP and NT-proBNP are glycosylated to varying degrees that can interfere with commercial immunoassays used to quantify NPs in clinical practice (14–16). The extent to which these immunoassays exhibit cross-reactivity to B-type natriuretic peptides as well as with their glycosylated and nonglycosylated forms is an important question to elucidate, as this may affect the clinical performance of the assays with implications for patient care. Furthermore, there are currently no formal efforts to standardize or maintain harmonization of BNP or NT-proBNP assays, primarily owing to the known differences in antibodies used and lack of a primary reference standard material.

As new heart failure therapeutic modalities are gaining regulatory approval, such as angiotensin receptor-neprilysin inhibitors (ARNI) it will be important not only to understand the mechanism of action of these drugs but also to ensure the natriuretic peptide assays used clinically are designed to measure the most relevant peptides. The purpose of this study was to comprehensively and systematically evaluate the specificity of BNP, NT-proBNP, and proBNP assays by determining cross-reactivity with a variety of glycosylated and nonglycosylated natriuretic peptides and NP fragments. This study built upon previously reported preliminary results from the International Federation of Clinical Chemistry (IFCC) Committee for Standardization of Markers of Cardiac Damage (14).

## Materials and Methods

### PLASMA SAMPLES

This study was approved by the institutional review board at Hennepin County Medical Center (Minneapolis, MN) and was developed in part by members of the Committee for Standardization of Markers of Cardiac Damage of the IFCC during and after their service on the Committee. Sample handling and storage conditions were consistent with published information regarding natriuretic peptide stability in human plasma (17, 18). EDTA plasma samples were collected from healthy donors in accordance with standard laboratory practices. Plasma was processed off cells within 1 h, aliquoted into

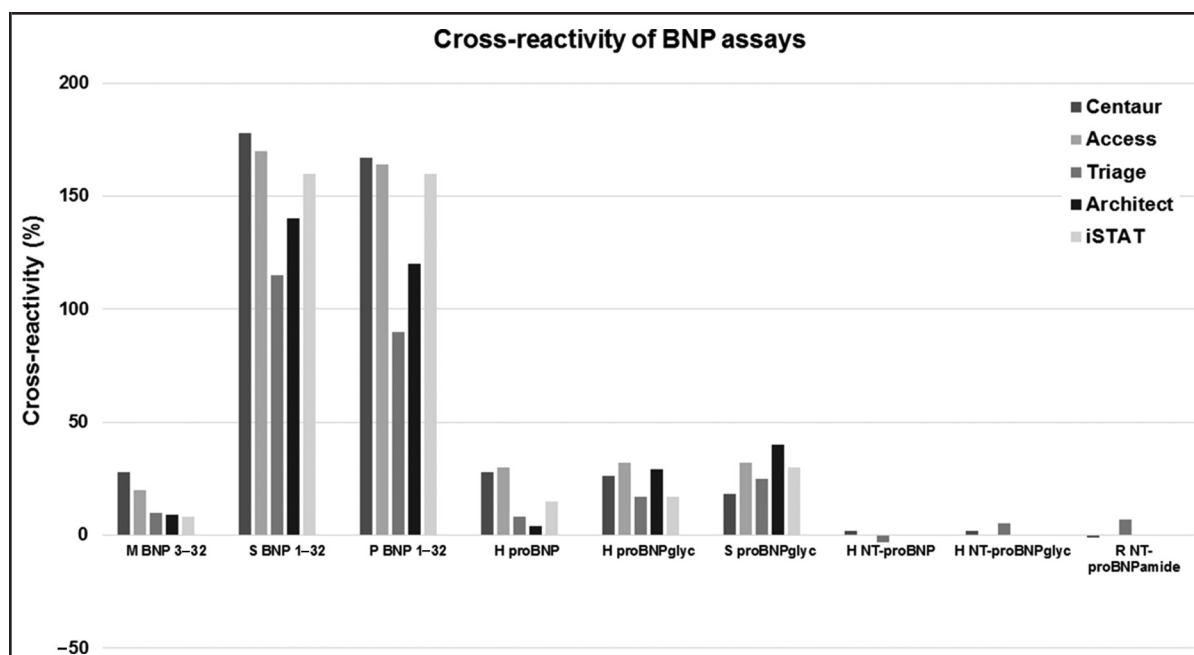
tubes containing a protease inhibitor cocktail (Roche Diagnostics), and stored at  $-70^{\circ}\text{C}$  until analysis. Once all assays were available, plasma samples were thawed at room temperature and gently mixed. The plasma was pooled and a protease inhibitor cocktail, thrombin inhibitor PPACK (Phe-Pro-Arg-Chloromethylketone, Bachem) and leupeptin (Bachem) were added. To ensure stability throughout the sample processing and analytical phases, we added additional protease inhibitor cocktail. Plasma pools were analyzed with all BNP, NT-proBNP, and proBNP assays to determine the baseline concentration. Pools were then individually spiked with 100 ng/L BNP, 300 ng/L proBNP, or 450 ng/L NT-proBNP peptides and reanalyzed with all assays. All measurements were performed in duplicate. The baseline BNP, NT-proBNP, or proBNP concentration was subtracted from each peptide-spiked pool measurement to determine cross-reactivity.

### BNP, NT-proBNP, AND proBNP PEPTIDES

The BNP peptides were obtained as synthetic BNP (aa 1–32; Shionogi, Peptide Institute), human recombinant BNP (aa 1–32; Scios), human recombinant BNP (aa 3–32; courtesy of John Burnett, Mayo Clinic, Rochester, MN). NT-proBNP peptides included human recombinant NT-proBNP (aa 1–76; HyTest), human recombinant glycosylated NT-proBNP (aa 1–76; HyTest), and synthetic amidated NT-proBNP; aa 1–76; Roche Diagnostics). Peptides for proBNP included HyTest human recombinant proBNP (aa 1–108; expressed in *Escherichia coli*), HyTest human recombinant glycosylated proBNP (aa 1–108; expressed in mammalian cells), and human recombinant glycosylated proBNP from Scios (aa 3–108).

### NATRIURETIC PEPTIDE ASSAYS

Five commercial BNP assays (see Table 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol63/issue1>) were evaluated and included: Abbott Architect and i-STAT, Alere Triage, Siemens Advia Centaur, and Beckman Coulter Access 2. Ten commercial NT-proBNP assays (see online Supplemental Table 2) included: Roche Diagnostics Elecsys 2010 Gen1 (no longer marketed; data not shown) and Gen2, Ortho-Clinical Diagnostics Vitros Eci, Response Biomedical RAMP, bioMerieux Vidas, Mitsubishi Kagaku Iatron Pathfast, Siemens Dimension, Immulite 2000, Immulite 2500, and Stratus CS. Research assays were provided by the manufacturer or by an independent researcher's laboratory (JPG) for proBNP and included: Bio-Rad (19), Goetze (20), and Biosite (Alere). All assays were performed according to manufacturers' guidelines and appropriate quality controls were analyzed with each batch of assays, with CVs ( $n = 10$  days) all  $<20\%$ . The inves-



**Fig. 1.** Cross-reactivity of BNP assays.

Five commercial BNP immunoassays and cross-reactivity (%) with BNP peptides (Mayo, M BNP 3-32; Scios, S BNP 1-32; Peptide Institute, P BNP 1-32), proBNP peptides (HyTest nonglycosylated proBNP, H proBNP; HyTest glycosylated proBNP, H proBNPglyc; Scios glycosylated proBNP, S proBNPglyc), and NT-proBNP peptides (HyTest nonglycosylated NT-proBNP, H NT-proBNP; HyTest glycosylated NT-proBNP, H NT-proBNPglyc; Roche nonglycosylated amidated NT-proBNP, R NT-proBNPamide).

tigational, research-use-only Bio-Rad assay was performed along manufacturer guidelines as described previously (19). The investigational, research-use-only Goetze assay was performed in the coauthor's laboratory as previously described (20). The investigational, research-use-only Biosite (Alere) proBNP assay was performed on a microfluidic protein chip. The capture antibody binds to the N-terminal end of proBNP and is designed to detect multiple forms of glycosylated and nonglycosylated proBNP. The assay was calibrated using glycosylated proBNP (HyTest) and calibrators were made by gravimetrically diluting the peptides into EDTA plasma. The dye-conjugated detection antibody is specific to the BNP region of proBNP. The selected proBNP-antibody-dye complex was captured on the microfluidic zones, unbound dye-antibody complex was washed from the detection lane into a waste chamber and the marker-antibody-dye complex bound on the capture zone via fluorescence (Triage Meter). The concentration of the complex was directly related to the amount of analyte in plasma.

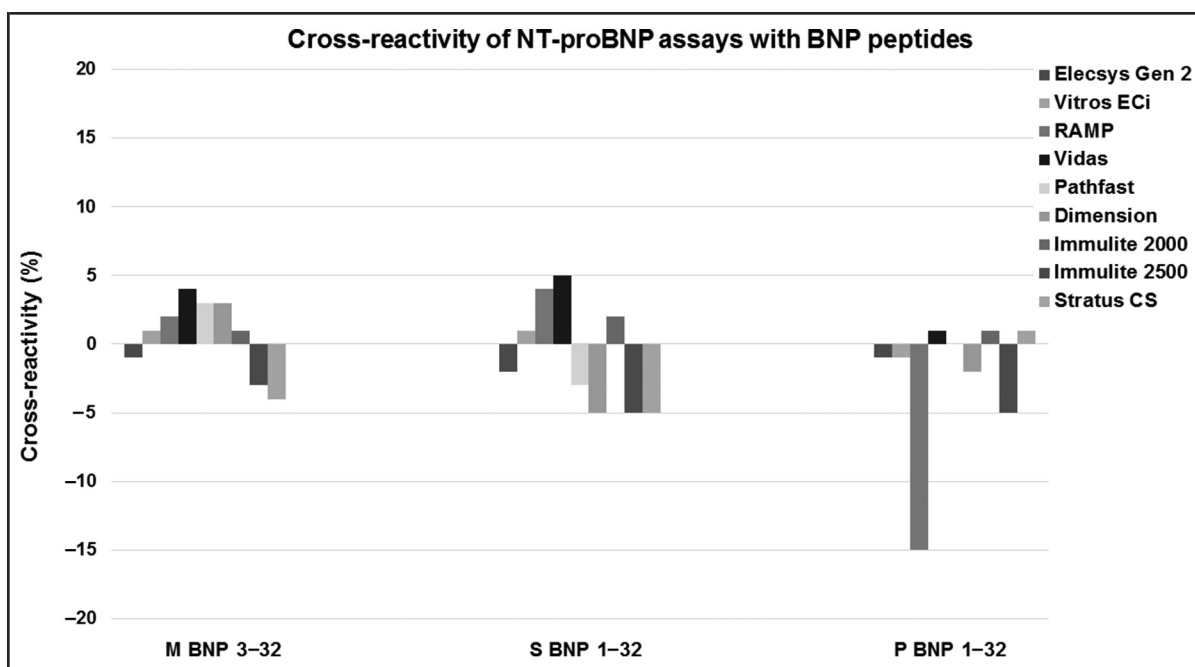
#### STATISTICAL PROCEDURES

For each assay and peptide combination, we used the mean of duplicate NP measurements on each sample

for all statistical analyses and comparisons. For plasma pools supplemented with a BNP, NT-proBNP, or proBNP peptide, we calculated the percentage recovery as the analyte concentration at the concentration of the peptide divided by the NP concentration of the unspiked sample, multiplied by 100. The criteria for the presence of a nonspecificity bias was defined as a cross-reactivity  $\geq 20\%$ .

#### Results

The cross-reactivity of BNP assays with BNP, proBNP, and NT-proBNP peptides is shown in Fig. 1. The BNP 3-32 peptide demonstrated the greatest cross-reactivity with the Siemens Centaur (28%) and Beckman Access (20%) assays. All BNP assays cross-reacted with the Scios BNP 1-32 peptide (115%–178%) and the synthetic Peptide Institute BNP 1-32 peptide (90%–167%). The Siemens Centaur, Beckman Access, and Abbott i-STAT BNP assays had similar cross-reactivity with the HyTest glycosylated and nonglycosylated proBNP peptides while the Alere Triage and Abbott Architect had 2-fold and 7-fold cross-reactivity with the glycosylated proBNP peptide compared to the nonglycosylated proBNP. All



**Fig. 2.** Cross-reactivity of NT-proBNP assays with BNP peptides.

Nine commercial NT-proBNP immunoassays and cross-reactivity (%) with BNP peptides (Mayo, M BNP 3-32; Scios, S BNP 1-32; Peptide Institute, P BNP 1-32).

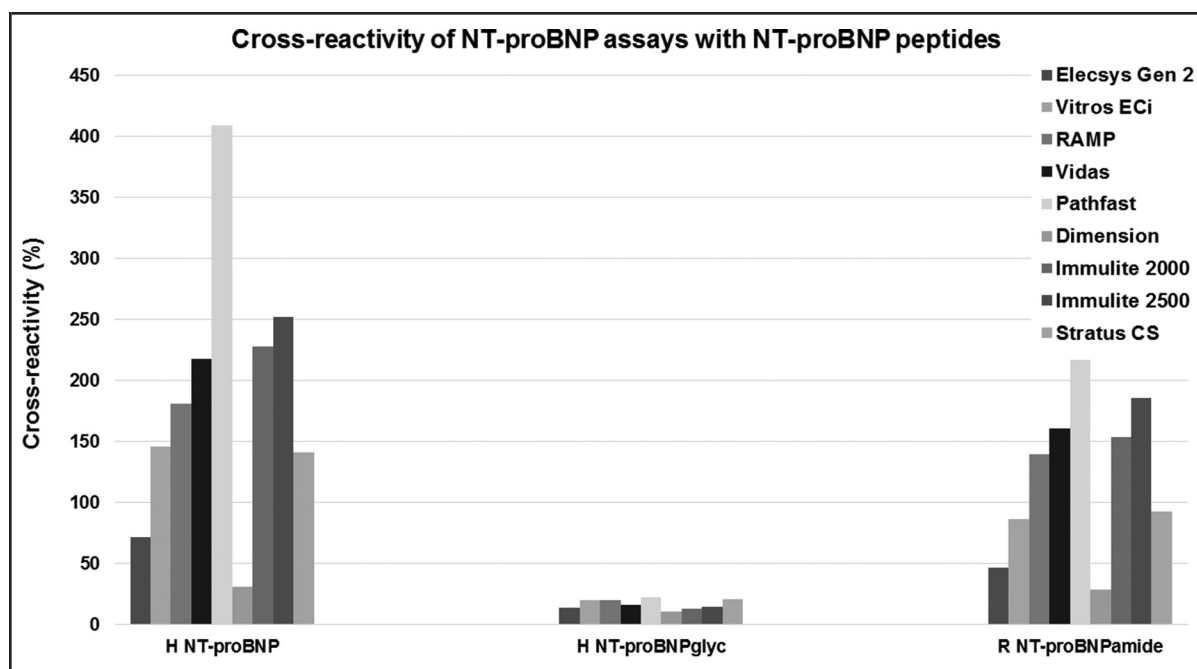
BNP assays had significant ( $\geq 20\%$ ) cross-reactivity with the Scios glycosylated proBNP peptide. None of the NT-proBNP peptides had cross-reactivity with any of the BNP assays.

The cross-reactivity of NT-proBNP assays with BNP, proBNP, and NT-proBNP peptides is shown in Figs. 2, 3, 4, respectively. There was minimal cross-reactivity of the NT-proBNP assays with the BNP peptides. The highest percent cross-reactivity was between the synthetic BNP 1-32 peptide and the Response Biomedical NT-proBNP RAMP assay ( $-15\%$ ). The Roche and bioMerieux NT-proBNP assays had similar antibody configurations but demonstrated different cross-reactivity with HyTest ( $72\%$  vs  $218\%$ ) and amidated Roche ( $47\%$  vs  $161\%$ ) NT-proBNP peptides, respectively. Differing cross-reactivity was also noted between the Pathfast and Vitros NT-proBNP assays as well as between the Siemens Dimension and Stratus NT-proBNP assays, each pair of which have identical antibodies, in response to the HyTest and Roche NT-proBNP peptides. None of the NT-proBNP assays demonstrated immunoreactivity with the HyTest (range:  $1\%$  to  $7\%$ ) or Scios (range:  $-2\%$  to  $3\%$ ) glycosylated proBNP peptides. All NT-proBNP assays recognized the human recombinant proBNP peptide with significant cross-reactivity, with the Siemens Dimension ( $16\%$  cross-reactivity) and Roche Elecsys 2 ( $30\%$ ) assays yielding the lowest cross-reactivity.

The percent cross-reactivity of 3 proBNP assays with BNP, proBNP, and NT-proBNP peptides is shown in Fig. 5. The Goetze proBNP assay was the only assay to demonstrate substantial cross-reactivity with any of the BNP peptides and exhibited  $26\%$  cross-reactivity only to the Mayo BNP (3-32) peptide. The other proBNP assays did not demonstrate significant immunoreactivity with the BNP peptides. The Biosite assay (nonglycosylated + glycosylated proBNP detection, calibrated with glycosylated proBNP) had similar percent cross-reactivity (range:  $55\%$  to  $77\%$ ) with all proBNP peptides at a target concentration of  $300$  ng/L. The proBNP assays also recognized the glycosylated Scios and HyTest proBNP peptides to a varying extent. The Goetze proBNP had the greatest cross-reactivity with glycosylated proBNP with  $426\%$  and  $324\%$  for Scios and HyTest peptides, respectively. The Bio-Rad assay had less immunoreactivity to the HyTest glycosylated proBNP peptide ( $15\%$ ) compared to the Scios glycosylated proBNP peptide ( $27\%$ ). None of the proBNP assays demonstrated significant cross-reactivity with any of the NT-proBNP peptides.

## Discussion

This study confirms there is significant cross-reactivity between proBNP peptides and BNP and NT-proBNP assays which are used in clinical practice, though the cross-reaction is largely variable among different assays.



**Fig. 3.** Cross-reactivity of NT-proBNP assays with NT-proBNP peptides.

Nine commercial NT-proBNP immunoassays and cross-reactivity (%) with NT-proBNP peptides (HyTest nonglycosylated NT-proBNP, H NT-proBNP; HyTest glycosylated NT-proBNP, H NT-proBNPglyc; Roche nonglycosylated amidated NT-proBNP, R NT-proBNPamide).

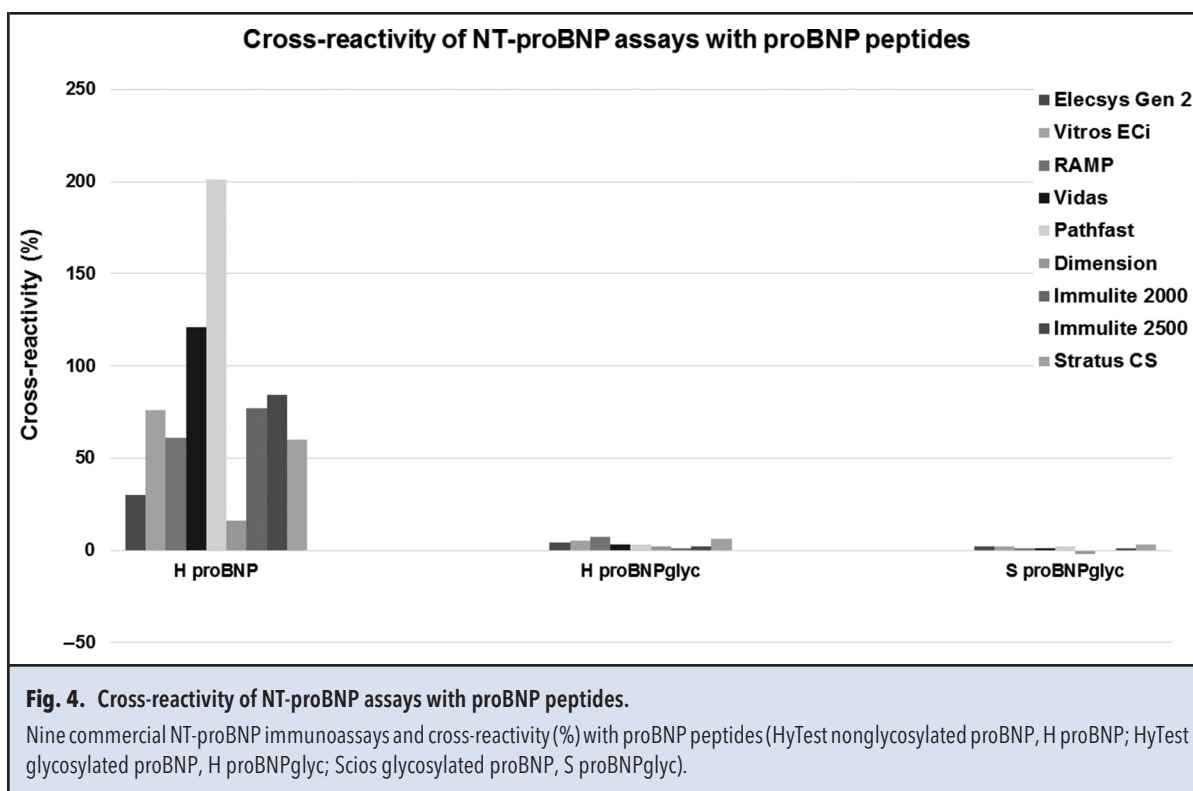
Our findings are unique in that they comprehensively and systematically demonstrate and confirm (a) there is no cross-reactivity between NT-proBNP peptides in BNP assays; (b) NT-proBNP assays do not detect BNP peptides or glycosylated proBNP-derived peptides, the major form seen in heart failure; and (c) proBNP assays are highly specific for the various forms of proBNP peptides that may have utility in elucidating peripheral processing of proBNP 1–108, particularly in heart failure patients and individuals with diabetes mellitus where glycosylation patterns are highly variable. These studies build upon the significant previous work conducted in this area (14–16).

The pathophysiological mechanisms behind natriuretic peptide release, breakdown, and clearance are complex but critical to understand to provide further insight into the heterogeneous spectrum of heart failure (21). The diverse nature of circulating natriuretic peptides and fragments makes analytical detection particularly challenging. Commercially available natriuretic peptide immunoassays exhibit large systematic differences between methods because the various assays cross-react to a differing extent with various BNP, NT-proBNP, and proBNP peptides.

All BNP assays demonstrate varying degrees of cross-reactivity with proBNP because the entire BNP 1–32 remains intact in the C-terminal portion of proBNP and

the assay antibodies recognize epitopes synergistic to both BNP and proBNP (22, 23). Shimazu et al. first elucidated the fact that the major form of BNP in plasma is not BNP 1–32 but instead is a truncated BNP 3–32 peptide produced following catalysis by the DPP IV enzyme (24). BNP assays cross-react with the BNP 3–32 peptide but also with proBNP peptides which are glycosylated and nonglycosylated. Thus, BNP assays are detecting all of these intact forms of the proBNP peptide as well as truncated BNP fragments. The present study demonstrates differing cross-reactivity in BNP assays even for assays that have identical antibody configurations, indicating there are likely other analytical factors which contribute to variation in BNP results.

We previously hypothesized the systematic differences between the various NP assays were largely a result of the cross-reactivity of the assays with glycosylated or nonglycosylated proBNP (14). proBNP undergoes post-translational glycosylation at Thr36, Ser37, Ser44, Thr48, Ser52, Thr58, and Thr71 and glycosylation in these regions is important for additional processing of proBNP into BNP and NT-proBNP (25). Glycosylation at Thr71 is of particular importance because it suppresses downstream processing of proBNP; thus, a majority of circulating proBNP is glycosylated at Thr71 while NT-proBNP Thr71 is not glycosylated. None of the NT-proBNP assays detect glycosylated forms of proBNP-

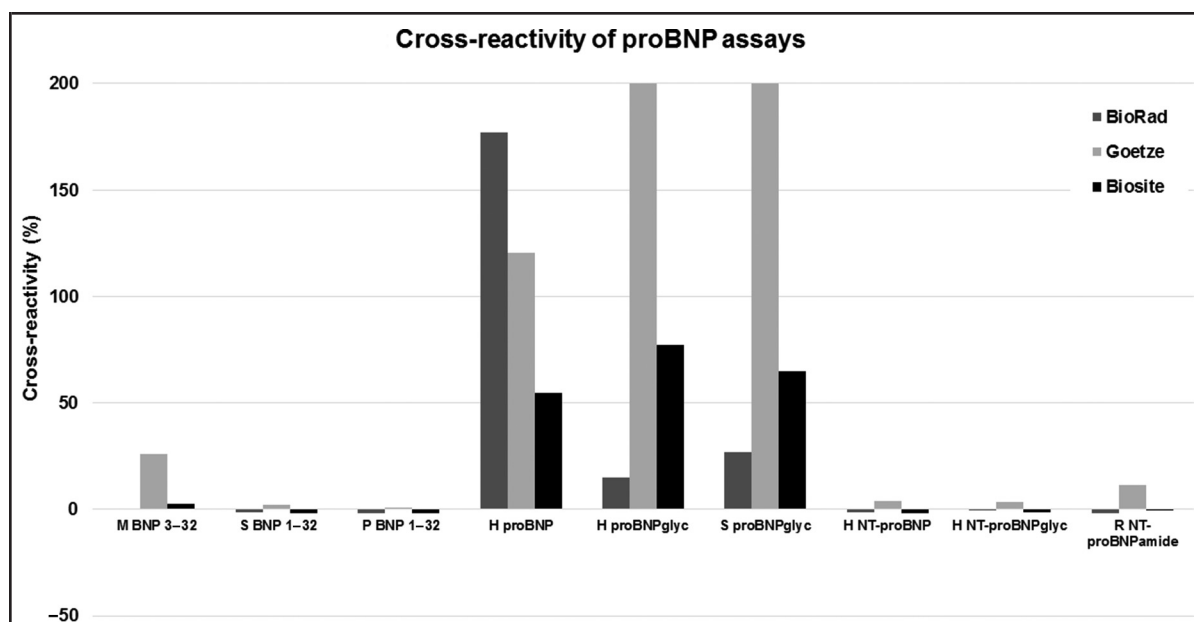


derived peptides because glycosylation of O-linked oligosaccharide almost completely inhibits signal antibody binding to the peptide (14). In contrast, there was recognition of glycosylated proBNP molecules with the Bio-Rad and Goetze proBNP assays. The Bio-Rad proBNP assay recognizes the processing or “hinge” region of the molecule that is specific to proBNP indicating, and confirmed with our data, that there is no cross-reactivity with BNP or NT-proBNP fragments (26). The Bio-Rad proBNP assay also additionally demonstrates cross-reactivity with glycosylated proBNP molecules, suggesting it may reflect the true amount of circulating nonglycosylated and glycosylated proBNP peptides. Additional studies are required to determine the clinical relevance of proBNP assays and the relationship between detecting glycosylated vs nonglycosylated proBNP.

The extent of glycosylation within individual samples has an impact on the analytical result. In previous studies, the Roche Elecsys Gen I detection antibody (aa 39–50) demonstrated poor cross-reactivity with glycosylated NT-proBNP and this was confirmed in our study (data not shown). A significant increase in plasma NT-proBNP concentrations was noted following deglycosylation, suggesting that this assay measures only 20% of circulating NT-proBNP which corresponds to the nonglycosylated form (15, 16). The Roche Elecsys NT-proBNP Gen II assay also uses antibodies directed to the

central glycosylated region (aa 42–46) and nearly all NT-proBNP assays used clinically use Roche NT-proBNP I or NT-proBNP II antibodies which recognize residues in this highly glycosylated region. In the present study, we have demonstrated NT-proBNP assays demonstrate minimal immunoreactivity with glycosylated peptides. This is because the antibodies used in the NT-proBNP assay are targeted to the regions of the NT-proBNP molecule that, when glycosylated, lose immunoreactivity. There are interindividual differences in NT-proBNP and proBNP glycosylation patterns in patients with and without heart failure and therefore current NT-proBNP immunoassays underestimate “true” NT-proBNP concentrations. Assessment of deglycosylated NT-proBNP concentrations also has been shown to improve the diagnostic and prognostic accuracy in patients with dyspnea, which suggests the antibody configurations used in NT-proBNP assays could be improved if glycosylated peptides were also detected (27).

Differences in assay calibration contribute to the differences noted between assays even with the same monoclonal antibody targets. Potential strategies to achieve harmonization may include utilizing an “in vitro-generated” peptide fragment as a calibrator that is representative of all secreted precursor molecules on a molar basis (20). This would avoid cross-reactivity and interference from the endogenous peptides. However, calibration



**Fig. 5. Cross-reactivity of proBNP assays.**

Three research-use-only proBNP immunoassays and cross-reactivity (%) with BNP peptides (Mayo, M BNP 3-32; Scios, S BNP 1-32; Peptide Institute, P BNP 1-32), proBNP peptides (HyTest nonglycosylated proBNP, H proBNP; HyTest glycosylated proBNP, H proBNPglyc; Scios glycosylated proBNP, S proBNPglyc), and NT-proBNP peptides (HyTest nonglycosylated NT-proBNP, H NT-proBNP; HyTest glycosylated NT-proBNP, H NT-proBNPglyc; Roche nonglycosylated amidated NT-proBNP, R NT-proBNPamide).

with an endogenous proBNP-derived peptide could be problematic because of the known differences between intact proBNP and proBNP fragments in those with and without cardiovascular disease, but cannot be ruled out as a possibility. In addition, it is not clear at present whether the reference material should be glycosylated or nonglycosylated.

We note the following limitations. First, this study was not designed to validate the analytical characteristics of each assay or to investigate the direct comparison between immunoassay results, but this type of assessment is proposed for future studies. Second, the results presented are from a single spiking and a single duplicate measurement. Thus it is potentially feasible that extreme results are the result of erroneous spiking or measurement. We did analyze plasma pools using higher concentrations of the peptides; however, because we found no statistical differences in the results these data were not included.

In conclusion, despite lack of standardized natriuretic peptide assays, clinical cardiovascular and heart failure guidelines recommend uniform cutpoints for BNP and NT-proBNP assays. This study demonstrates there are opportunities to improve standardization and harmonization efforts for natriuretic peptide assays and ultimately to improve clinical care for heart failure pa-

tients, particularly on the advent of new pharmacologic agents.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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