N-terminal Pro–Atrial Natriuretic Peptide Measurement in Plasma Suggests Covalent Modification

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BACKGROUND: The N-terminal fragment of cardiac-derived pro–B-type natriuretic peptide is a glycosylated polypeptide. It is unknown whether N-terminal pro–atrial natriuretic peptide (proANP) fragments are also covalently modified. We therefore evaluated the clinical performance of 2 distinctly different proANP assays and compared the methods with respect to overall assay performance, as well as the differences between the proBNP-derived peptides and the methods used is not straightforward (3). For the proANP-derived peptides, little is known about post-translational modifications, and their clinical measurement has not yet been challenged with regard to assay calibration. We therefore examined 2 different proANP assays and compared the methods with respect to both their measurements and overall clinical performance. Finally, we evaluated the primary proANP structure in plasma. An automated assay has also been launched for the N-terminal fragment of the homologous atrial natriuretic peptide (ANP) prohormone (1).

CONCLUSIONS: Our data suggest that N-terminal proANP fragments in patient plasma differ from the calibrator peptides used but that the difference does not affect ROC curves in an elderly cohort of patients with mild to moderate heart failure. We suggest that human N-terminal proANP fragments can be covalently modified.

Plasma measurements of cardiac-derived peptides and their prohormones are established markers of cardiac dysfunction, with normal concentrations excluding a diagnosis of heart failure. Increased plasma concentrations also contain prognostic information after a myocardial infarction has occurred and in patients with an established diagnosis of heart failure. Automated assays are available in routine hospital laboratories for B-type natriuretic peptide (BNP)5 and the N-terminal precursor fragment in plasma. An automated assay has also been launched for the N-terminal fragment of the homologous atrial natriuretic peptide (ANP) prohormone (1).

Although clinical studies have not detected major differences between the proBNP-derived peptide methods with respect to overall assay performance, assay standardization still presents a considerable challenge. New data have revealed complex cardiocyte processing of proBNP, including variable exo- and endoproteolytic cleavages, as well as O-glycosylation in the midregion (MR) (2). Consequently, calibration of the methods used is not straightforward (3). For the proANP-derived peptides, little is known about post-translational modifications, and their clinical measurement has not yet been challenged with regard to assay calibration. We therefore examined 2 different proANP assays and compared the methods with respect to both their measurements and overall clinical performance. Finally, we evaluated the primary proANP structure in a search for a molecular explanation for the differences in the concentration values produced by the 2 methods.

We examined a cohort of 474 elderly patients who presented to the primary physician with symptoms of heart failure. We have previously reported on this patient cohort (4, 5). This group of patients is notable for having several comorbidities and only mild to moderate heart failure. This clinical setting is similar to the scenario in which these biomarkers are usually applied and for which other tests are not readily available. For immunoassays, we used an automated MR-proANP assay that is based on monoclonal antibodies raised against sequences 53–72 (PEVPPWTGEVSPAQRDGGAL) and

5 Nonstandard abbreviations: BNP, B-type natriuretic peptide; ANP, atrial natriuretic peptide; MR, midregion; PIA, processing-independent assay; AUC, area under the ROC curve.
73–90 (GRGPWDSSDRSALLKSKL) in the proANP structure. The assay validation, in which the proANP fragment 53–90 was used as the calibrator peptide, has previously been described (1). For comparison, we used a new processing-independent assay (PIA) that we developed in our laboratory [for reviews of the analytical principle, see (6, 7)]. This assay detects the C terminus of proANP fragment 1–16 after enzymatic cleavage. This assay allows variable and unknown processing to be bypassed and reports on all possible fragments secreted into the circulation, irrespective of endoproteolytic cleavages and amino acid modifications. The calibrator peptide is proANP 1–16. The 2 assays were compared by means of a difference plot (Bland–Altman plot), and clinical performance was assessed by generating ROC curves and calculating the area under the ROC curve (AUC) for different clinical outcomes.

The difference plot for the 2 methods is shown in Fig. 1A. Despite a linear regression analysis showing a good correlation \( r = 0.85; P < 0.0001 \), the PIA measured markedly higher proANP concentrations than the MR-proANP assay (mean difference, 663 pmol/L; SD, 478 pmol/L). Thus, endogenous proANP-derived peptides in plasma may differ in molecular structure from the calibrator peptide used in the MR-proANP assay. In contrast, the measured fragment after preanalytical enzymatic treatment is the same as that used for the assay calibrator in the PIA method. On the other hand, the 2 methods were similar in clinical performance with respect to detecting left ventricular dysfunction (defined as a left ventricular ejection fraction \(<40\%\)) as measured by the AUC [proANP PIA, 0.71 (95\% CI, 0.63–0.79); MR-proANP assay, 0.74 (95\% CI, 0.66–0.81); \( P = 0.32 \)] (Fig. 1B). Notably, only 55 patients displayed a reduced left ventricular function, whereas 419 did not. The prognostic ability in predicting cardiovascular mortality (during a 10-year follow-up with no dropouts) revealed AUC values of 0.66 (95\% CI, 0.60–0.71) for the proANP PIA and 0.69 (95\% CI, 0.63–0.74) for the MR-proANP assay \( (P = 0.08 \) for the comparison of the 2 assays; Fig. 1C). In the entire cohort, 119 patients died of cardiovascular causes, and 355 died of other causes. The data suggest that the proANP detected in patient plasma differed from the calibrator peptides. This finding did not affect the results obtained from standard ROC curves and in Cox proportional hazard regression analyses (Table 1) in a cohort of patients with mild to moderate heart failure.

To elucidate the possible plasma proANP forms, we evaluated the primary structure with software that predicts amino acid modifications. Although this software generates only hypotheses, it is notable that the programs suggested O-glycosylation in the proBNP molecule (see the Data Supplement that accompanies Fig. 1. (A), Difference plot of MR-proANP assay and the proANP PIA (n = 474). Note the mean difference of 663 pmol/L (solid line; broken lines indicate the 95\% confidence limits). (B), Ability to detect a reduced left ventricular ejection fraction as defined by echocardiography. (C), ROC curves for predicting cardiovascular death over a 10-year follow-up. The analyses revealed no significant differences between the 2 proANP assays.
the online version of this Brief Communication at http://www.clinchem.org/content/vol57/issue9) long before biochemical evidence confirmed it. The NetOGlyc and YinOYang software both predicted glycosylation sites in proANP, for residues covering most of the N-terminal 1–98 fragment; however, the serine residues at positions 79 and 80 (the location of one of the epitopes for the MR-proANP assay) were predicted with high probability as potential O-glycosylation sites. In line with our results for the difference plot (Fig. 1A), that prediction could explain the substantial differences between the 2 assays in measured concentrations. Detection with the MR-proANP assay may be affected by glycosylation and thus may underestimate endogenous concentrations in plasma. In contrast, the PIA measures an epitope in a region with no suggested amino acid modifications. Interestingly, a threonine residue at position 95 is also predicted to be O-glycosylated (see Table 1 in the online Data Supplement). Whether these potential modifications are involved in endoproteolytic proANP maturation and the release of bioactive ANP, as is the case for proBNP maturation (8), remains to be examined.

Thus far, N-terminal proANP fragments have not been reported to be glycosylated in plasma or in proANP produced in vitro by HEK293 or murine HL-1 cells (9). The elution profile of human proANP with standard gel chromatography points toward unidentified modifications, however, and these findings have been the basis for suggestions that N-terminal proANP oligomerizes (10). In parallel with the different proBNP forms, it seems more likely that the N-terminal proANP fragments are modified monomers without any secondary or tertiary structure (11). We suggest that posttranslational modification of proANP be investigated, because differences in calibration could lead to different assay performance characteristics for certain populations. Although the present ROC analyses revealed no differences with respect to detecting a reduced cardiac function or a risk of cardiovascular mortality in elderly patients with mild to moderate heart failure (Fig. 1, B and C; Table 1), these data should be interpreted with caution. The use of ROC curves is based on a dichotomous division of the data, whereas numerical changes over the entire concentration range will not be picked up by such analyses. It is reasonable to assume, however, that the numerical values of plasma measurements would also be used in clinical practice, with higher values suggesting more-severe disease. It is our impression that measurements of cardiac-derived peptides are already used as indirect measures of clinical worsening. ROC curves are not sensitive to such changes. We speculate that any differences will become more pronounced as cardiac failure worsens, possibly leading to less efficient processing of the prohormones. Using proANP measurement as a molecular marker within heart failure must take into account the possibility that the endogenous peptides might

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≥75 years</td>
<td>1.80 (1.21–2.67)</td>
<td>0.003</td>
<td>1.95 (1.31–2.91)</td>
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<td>Male sex</td>
<td>0.69 (0.46–1.03)</td>
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<td>0.73 (0.49–1.10)</td>
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<td>NYHA3 class III</td>
<td>1.79 (1.10–2.90)</td>
<td>0.02</td>
<td>1.87 (1.14–3.07)</td>
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<td>EF &lt;40%</td>
<td>2.00 (1.26–3.19)</td>
<td>0.003</td>
<td>2.19 (1.38–3.48)</td>
<td>0.0009</td>
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<tr>
<td>IHD</td>
<td>0.80 (0.54–1.18)</td>
<td>0.26</td>
<td>0.83 (0.56–1.23)</td>
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<tr>
<td>BMI &gt;27 kg/m²</td>
<td>0.88 (0.60–1.30)</td>
<td>0.52</td>
<td>0.89 (0.60–1.31)</td>
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<tr>
<td>eGFR &lt;60 mL·min⁻¹·(1.73 m²)⁻¹</td>
<td>1.40 (0.93–2.12)</td>
<td>0.11</td>
<td>1.34 (0.88–2.02)</td>
<td>0.17</td>
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<td>Diabetes</td>
<td>1.99 (1.33–2.99)</td>
<td>0.0009</td>
<td>2.01 (1.34–3.00)</td>
<td>0.0007</td>
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<tr>
<td>Hb &lt;12.0 g/dL</td>
<td>1.66 (0.89–3.09)</td>
<td>0.11</td>
<td>1.64 (0.88–3.08)</td>
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<tr>
<td>MR-proANP assay Q4</td>
<td>2.42 (1.61–3.64)</td>
<td>&lt;0.00011</td>
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<td>—</td>
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<tr>
<td>proANP PIA Q4</td>
<td>—</td>
<td>—</td>
<td>1.81 (1.13–2.89)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

a Model I: clinical variables analyzed together with the fourth quartile (Q4) of concentrations measured with the MR-proANP assay.
b Model II: various clinical variables analyzed together with the fourth quartile (Q4) of concentrations measured with the proANP PIA.
c NYHA, New York Heart Association class; EF, ejection fraction; IHD, ischemic heart disease; BMI, body mass index; eGFR, estimated glomerular filtration rate (Modification of Diet in Renal Disease Study equation formula); Hb, hemoglobin.
differ as a function of cardiac status. The problem is well known for other hormones. For instance, the measurement of thyroid-stimulating hormone is complicated by altered glycosylation as a function of the state of the thyroid (12), and gastrin is affected by disease-dependent molecular heterogeneity in the plasma (13, 14).

In conclusion, our data suggest that proANP fragments detected in patient plasma can differ from the calibrator peptide and that the differences are not detected by ROC curves in a cohort of elderly patients with mild to moderate heart failure. Possible posttranslational modification of N-terminal proANP fragments should be investigated.

**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.

**References**

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Previously published online at DOI: 10.1373/clinchem.2011.166330