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A multimarker approach to diagnose and stratify heart failure

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ABSTRACT

Background: We have previously demonstrated that circulating NT-proBNP is truncated at the N and C termini. Aims of this study are three-fold: firstly to determine whether the NT-proBNP levels correlate with NYHA functional classes when measuring with different antibody pairs; secondly to evaluate the diagnostic potential of ProBNP and; thirdly to investigate whether combining NT-proBNP assays with or without ProBNP would lead to better diagnostic accuracies.
Methods: Plasma samples were collected from healthy controls (n = 52) and HF patients (n = 46). Customized AlphaLISA® immunoassays were developed and validated to measure the concentrations of proBNP and NT-proBNP (with antibodies targeting 13–45, 13–76, 28–76). The diagnostic performance and predictive value of proBNP and NT-proBNP assays and their combinations were evaluated.
Results: Plasma proBNP assay showed acceptable diagnostic performance. NT-proBNP$_{13–76}$ assay is useful in diagnosing and stratifying HF patients. The diagnostic performance of NT-proBNP$_{13–76}$ demonstrated improvement over commercial NT-proBNP tests. The combination of NT-proBNP$_{13–76}$ with NT-proBNP$_{28–76}$ assays gave the best diagnostic assay performance.
Conclusion: Our results demonstrate that while there is major heterogeneity in circulating NT-proBNP, specific epitopes of the peptides are extraordinarily stable, providing ideal targets for clinically useful diagnostic assays. Future new clinical diagnostic clinical trials should include a multimarker approach rather than using a single marker to diagnose HF.

1. Introduction

Heart failure (HF) is the leading cause of death worldwide, with 17.3 million deaths per year [1]. The incidence of HF is projected to increase by 2030 to 23.6 million due to an aging and a growing population [1]. Current medical therapy including angiotensin converting enzyme (ACE) inhibitors, beta blockers and mineralocorticoid receptor antagonists may slow the progression of disease and prolong survival [2], but are generally started late in the disease trajectory. Prevention of HF progression by early detection therefore becomes important in the reduction of HF disease burden.

Echocardiography is the single most useful diagnostic test to evaluate a patient with suspected HF, including the evaluation of left ventricular (LV) systolic and diastolic dysfunction [2–4] to guide patient management. In addition, cardiac-specific biomarkers including plasma natriuretic peptides (N-terminal proBNP and BNP) are useful when the diagnosis is unclear and in circumstances where an echocardiogram cannot be performed in a timely fashion [5,6]. Ventricular wall stress and ischemia stimulate the cardiomyocytes to secrete proBNP [1–108 AA] [7], which is cleaved by furin (a ubiquitous serine protease), corin (cardiac serine protease) or other currently unknown proteases to BNP (77–108 AA) and NT-proBNP (1–76 AA) [8]. Plasma levels of BNP and NT-proBNP increase with disease severity and are associated with an adverse prognosis in patients with HF [5,6,9]. A recent review [10] summarized the diagnostic performance of the current Roche NT-proBNP assay approved by the FDA (Roche Diagnostics, Basel, Switzerland) and it showed that the pooled sensitivity and specificity from 20 clinical studies were 80% and 61%, respectively, at the optimal cut points. BNP-32 and proBNP-108 (with and without glycosylation) have been detected in plasma from healthy controls and HF patients with atrial fibrillation [11]. We and others have identified various molecular forms of BNP and NT-proBNP in plasma including the presence and absence of glycoslation and truncated forms [12,13]. This molecular heterogeneity of NT-proBNP may be a confounding issue in measurements depending on the assay, and could affect diagnostic cut-offs, and reduce the ability to detect serial changes when using these peptide

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levels. Furthermore, recent studies have demonstrated that not only NT-proBNP and BNP levels are increased in the plasma of patients with HF, but that the levels of the precursor molecule proBNP are elevated and correlate with NYHA functional class [11,14]. Indeed, the antibodies of the current BNP and to a lesser extent NT-proBNP immunoassays cross-react with proBNP [13,15]. A recent study has also confirmed previous findings by demonstrating that the antibody targeting BNP cross reacts with 50% of the total proBNP in plasma, but there was only 5% cross reactivity between the proBNP antibody and the NT-proBNP molecule [16]. Furthermore, when BNP and proBNP were measured in parallel, elevated levels of both of these peptides was a strong indicator for the development of HF [17]. Despite the benefits of measuring proBNP in circulation to diagnose HF, plasma proBNP is not used in clinical practice [18] and warrants further investigation.

We have previously demonstrated that using antibody pairs targeting different parts of the full length NT-proBNP molecule (13–45 a.a.r, 13–76 a.a.r and 28–76 a.a.r) are present in plasma samples from HF patients facilitating HF diagnosis [12]. The aims of this study are three-fold: firstly to determine whether the plasma measurements of NT-proBNP levels using different antibodies pairs vary according to NYHA functional class (I–III), secondly to evaluate the diagnostic potential of plasma ProBNP and thirdly to determine whether by combining NT-proBNP levels or without ProBNP improves diagnostic accuracy compared to measuring full length NT-proBNP.

2. Materials and methods

2.1. Participants and sample collection

The study complies with the 1975 Declaration of Helsinki. The project was approved by the University of Queensland Medical Ethical Institutional Board, Mater Medical Ethical Review Board, Royal Brisbane and Women's Hospital Research Governance. Two groups of healthy participants with no underlying cardiovascular diseases were recruited: young participants aged between 18 and 39 (years) and middle age group of participants aged > 40 years. Signed informed consents were obtained from all participants before sample collection. Cardiologists based on clinical symptoms, categorized HF patients according to their NYHA functional class (refer to Table 1). Volunteers were of European, Asian and Indian descent. Volunteers were asked to refrain from eating and drinking (except plain water) two hours prior to sample collection. Blood samples were collected in EDTA tubes (Greiner VACUETTE® # 454023, Greiner Bio-one, Graz, Austria) and centrifuged at 500 g at 24 °C for 15 min to separate plasma from blood cells. Each plasma aliquot was stored at −80 °C until analysis.

2.2. Biotinylation of proBNP and NT-proBNP monoclonal antibodies and coupling NT-proBNP monoclonal antibodies to acceptor beads for AlphaLISA® immunoassays

A detailed description of the antibodies (manufacturer, immunogenicity, cross-reactivity between natriuretic peptides) used for the AlphaLISA® immunoassays is listed in Supplementary Table 1.

ProBNP monoclonal antibodies (mAbs) 50E1 and NT-proBNP mAbs 28F8 and 5B6 were biotinylated with N-hydroxysuccinimido-ChromaLink-biotin (2 mg/ml) (Product-No: 9007-105K, Solulink, CA, USA) at molar ratio of 30:1 with an incubation time of 2 h. Unbound biotinylated proBNP and NT-proBNP monoclonal antibodies were removed using Zeba spin desalting columns (Product-No: 89882, Thermo Scientific Pierce, IL, USA) and biotinylated antibodies were stored at 4 °C.

AlphaLISA® acceptor beads (Product-No: 6772003, Perkin Elmer®, Waltham, MA, USA) were mixed with 250 μL phosphate buffer solution and centrifuged at 16000 ×*g* for 15 min and the supernatant was discarded. For coupling of NT-proBNP monoclonal antibodies 11D1 and 18H5 (0.1 mg, respectively) were added to acceptor beads, 1.25 μL of 10% Tween-20, 25 μg of $NaBH_3CN$ and PBS (0.13 M) and incubated for 24 h at 37 °C. After that, 10 μL of carboxy-methoxylamine (CMO) was added to the reaction and incubated for 1 h at 37 °C. Conjugated NT-proBNP monoclonal antibodies were collected by centrifugation at 16000 ×*g* for 15 min and the supernatants were discarded. The acceptor beads were washed twice in 1 mL of 0.1 M Tris–HCl (pH 8). Resuspension of conjugated NT-proBNP monoclonal antibodies to the acceptor beads in 1X PBS-0.05% Proclin 300 were performed before sonication. The purified NT-proBNP monoclonal antibodies conjugated to the acceptor beads were stored at 4 °C until analysis.

Table 1
Clinical features of patients presenting with heart failure (HF). HF patients were classified according to the New York Heart Association functional classification. Two groups of healthy controls were selected to determine the influence of age on the circulating level of ProBNP and NT-proBNP fragment concentrations.

Parameter	HF patients (n = 46)	Healthy controls (n = 52)
Age (years)	70 (26–53)	40 (21–49)
>18 and <40	–	17
>40	46	35
Gender ratio (male: female)	1.1	2.1
NYHA 1	11	–
NYHA 2	15	–
NYHA 3	20	–

2.3. In-house development of AlphaLISA® immunoassays for the detection of proBNP, NT-proBNP$_{13–76}$, NT-proBNP$_{13–45}$, and NT-proBNP$_{28–76}$

The AlphaLISA® assay technology (PerkinElmer®, USA) is a homogeneous, bead-based sandwiched immunoassay and offers many advantages over traditional Enzyme-Linked Immunosorbent Assay (ELISA) including higher sensitivity (1 pg/mL), a requirement for only a low volume of samples, and enhanced reproducibility due to no wash steps [19]. In the presence of analytes (proBNP or NT-proBNP), the streptavidin coated donor beads and acceptor beads come into close proximity. Excitation of the donor beads will promote the release of oxygen singlet molecules thereby triggering a cascade of energy transfer to the acceptor beads, resulting in a sharp peak of light emission at 615 nm. NT-proBNP immunoreactivity were measured by using three NT-proBNP assays targeting different epitopes of the NT-proBNP molecule. The AlphaLISA® immunoassays for detecting proBNP and NT-proBNP in plasma were designed by targeting two different regions on proBNP and NT-proBNP molecules. The immunoassay nomenclature was based on the number of the first amino acid that the capture antibody binds to on the NT-proBNP molecule and the last amino acid that the detection antibody binds to on the NT-proBNP molecule of 1–76 amino acids: NT-proBNNP$_{13–45}$ (18H5$_{13–20}$ and 11D1$_{28–45}$); NT-proBNP$_{28–76}$ (11D1$_{28–45}$ and 28F8$_{67–76}$); NT-proBNP$_{13–76}$ (18H5$_{13–20}$ and 28F8$_{67–76}$).

For the quantification of proBNP and NT-proBNP, a 12-point standard curve was generated using either the proBNP analyte (Product-No: NBC1-18491, Novus Biologicals®, UK) or NT-proBNP analyte (Product-No: 8NT2, Hytest Ltd, Finland) in pooled plasma from five healthy volunteers (to match the sample matrix) at a 1:1 ratio with HiBlock immunoassay buffer (PerkinElmer, Inc., USA). All the assays were performed in a 10 μL reaction volume (sample volume was 1 μL). For all immunoassays, the end concentration of acceptor beads and biotinylated antibodies were 10 μg/mL and 1 nM, respectively.

Samples were loaded in triplicates in a 384 well ProxiPlates™ (Perkin Elmer®, Waltham, MA, USA). In brief the assay protocol for proBNP is as follows: firstly add acceptor beads to the sample and incubate for 30 min at room temperature (RT), followed by the addition of biotinylated proBNP and incubate for 1-hour. The final step is to add streptavidin donor beads, and incubate for 30 min at RT in the dark. Similarly, to determine the NT-proBNP concentrations in plasma, biotinylated antibody and acceptor beads were added to the samples and incubated for 1 h at RT followed by 30 minute incubation in the dark upon addition of streptavidin donor beads (final concentration 40 μg/mL). The only exception to the manufacturer protocol was to reduce total reaction volume from 50 μL to 10 μL. All experiments were performed by two trained individuals located in The University of Queensland Diamantina Institute.

2.4. Analytical performance of the in-house developed ProBNP and NT-proBNP AlphaLISA® immunoassays

2.4.1. Analyte recovery of proBNP, NT-proBNP$_{13–76}$, NT-proBNP$_{13–45}$ and NT-proBNP$_{28–76}$ immunoassays

To evaluate the suitability of AlphaLISA® immunoassays for measuring proBNP and NT-proBNP, two known concentrations of commercially available recombinant proBNP and NT-proBNP were spiked separately in pooled plasma collected from healthy controls (n = 5). Spiked and non-spiked pooled plasma were measured in the same AlphaLISA® immunoassay. The percentage recovery of the two spiked plasma samples was calculated with reference to corresponding un-spiked pooled plasma in a single AlphaLISA® immunoassay, using the following equation [20]

$$\text{Percentage recovery} = \frac{\text{Calculated concentration of analyte in spiked samples} - \text{concentration of analyte in unspiked samples}}{\text{Known concentration of spiked analyte}} \times 100.$$

2.4.2. Intra-and inter-assay coefficient of variation of proBNP, NT-proBNP$_{13–76}$, NT-proBNP$_{13–45}$ and NT-proBNP$_{28–76}$ immunoassays

To determine intra- and inter-assay variations, triplicates of plasma samples from 46 healthy controls and 52 HF patients were analyzed in one AlphaLISA® proBNP and NT-proBNP immunoassays and three independent AlphaLISA® proBNP and NT-proBNP immunoassays, respectively [21]. Intra- and inter-assay variations were expressed by intra- or inter-assay coefficient of variation (%CV). %CV was calculated using the following equation:

$$\%CV = (\text{Mean of SD}) \div (\text{Mean}) \times 100\%.$$