The Brain Natriuretic Peptide (BNP) Precursor is the Major Immunoreactive form of BNP in Patients with Heart Failure

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Abstract

Background: Heart failure (HF) diagnosis often relies on peptides originating from the BNP precursor, including BNP, proBNP, and the NT-proBNP, serving as key markers. However, there remains ambiguity regarding the specific forms of these peptides present in the bloodstream and their detectability through existing assays. **Objective:** To increase our understanding and knowledge of the circulating forms of BNP peptides in HF and their implications for diagnostic and prognostic assessment. **Study design:** An analytical cross-sectional study. **Place and Duration:** This study was conducted in The Indus Hospital and Health Network Karachi from March 2023 to March 2024. **Methodology:** In this research, we developed innovative assays for detecting NT-proBNP, proBNP, and BNP using specific monoclonal antibodies designed for their recognition. These antibodies were thoroughly tested in dual-site-combinations by using time-resolved immunoassays. We employed synthetic antigens and recombinant antigens, as well as plasma samples from HF patients. Furthermore, we utilized gel filtration fast protein liquid chromatography (FPLC) to analyse proBNP and related molecules in both pre- and post-protein fractionated HF plasma extracts and samples, using Sep-Pak C18 cartridges. **Results:** Our investigations demonstrated specific detection limits for proBNP, BNP, and NT-proBNP assays, measured at 0.4, 3, and 10 ng/L, respectively. Following analyses using gel filtration-FPLC, distinct peaks emerged, with one each for NT-proBNP (25 kDa) and proBNP (37 kDa), and two for BNP immunoreactivity. Particularly noteworthy was the finding that in patient plasma, the molar concentration of NT-proBNP exceeded that of proBNP by almost tenfold. The mean proBNP:BNP ratio in patient plasma was calculated at 6.3, with variations ranging from 1.8 to 10.8. **Conclusions:** Our findings highlight proBNP as the principal BNP-immunoreactive form circulating in human blood. Moreover, we underscore the influence of sample handling techniques and assay methodologies on determining the proBNP:BNP ratio in plasma samples, emphasizing the importance of standardized protocols in peptide measurement.

Keywords: **Heart Failure Biomarkers, Probnp Detection Assays, NT-Probnp Quantification, Peptide Immunoreactivity, Plasma Sample Analysis.**

Introduction

HF represents a significant concern of public health worldwide, with an estimated prevalence of over 64 million individuals affected globally [1]. This syndrome encompasses a spectrum of clinical manifestations stemming from the heart's inability to pump blood efficiently, resulting in systemic congestion and impaired tissue perfusion [2]. Despite advancements in therapeutic interventions, HF remains associated with substantial morbidity, mortality, and healthcare costs. Therefore, accurate and timely diagnosis is paramount for optimizing patient management and improving clinical outcomes [3].

Biomarkers have emerged as very beneficial tools in the formulation of a diagnosis, and monitoring of the patients with HF [4]. Among the mentioned biomarkers, peptide derived from the BNP precursor have garnered significant attention due to their close association with cardiac function and HF pathophysiology [5]. These peptides include proBNP, BNP, and the NT-proBNP, each offering unique insights into the hemodynamic stress and myocardial dysfunction characteristic of HF [6].

Despite their clinical utility, several challenges persist regarding the measurement and interpretation of BNP

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peptides in HF [7, 8]. A fundamental issue pertains to the various forms in which these peptides circulate in the bloodstream, and the extent to which current assays capture these different forms accurately [9]. Additionally, factors such as sample handling techniques, assay methodologies, and patient-specific variables may influence the reliability and reproducibility of BNP peptide measurements, further complicating their clinical interpretation [10, 11].

Addressing these challenges requires a comprehensive knowledge of the structural as well as the immunological characteristics of NT-proBNP, BNP, and proBNP, along with the development of robust assay methodologies capable of discerning between different peptide forms [12, 13]. In this context, our study aims to fill existing knowledge gaps by employing innovative assay designs and analytical techniques to characterize BNP peptides in plasma samples.

Specifically, we utilized a panel of MAbs targeting NT-proBNP and BNP epitopes to develop sensitive and specific assays for proBNP, NT-proBNP, and BNP detection. These assays were meticulously validated using recombinant or synthetic antigens, as well as plasma samples sourced from HF patients. Furthermore, gel filtration FPLC was employed to provide additional insights into the molecular composition of proBNP and related molecules in HF patients' samples.

Through our comprehensive approach, we aim to increase our understanding and knowledge of the circulating forms of BNP peptides in HF and their implications for diagnostic and prognostic assessment. Ultimately, our findings have the potential to inform the refinement of clinical practice guidelines, improve risk stratification strategies, and facilitate personalized therapeutic interventions in HF management.

Methodology

In this study, synthetic human BNP with 97% purity and human recombinant NT-proBNP and proBNP with 98% purity were obtained. Synthetic peptides corresponding to specific sequences of BNP and NT-proBNP were also acquired. All the chemicals utilized were of analytical grade. MAbs targeting NT-proBNP and BNP epitopes were generated using standard hybridoma cell line techniques.

We conducted a sandwich immunofluorescent assay (IFA) employing biotinylated monoclonal antibodies (MAbs) for capturing and MAbs labeled with a stable europium (III) chelate for detection. The assay involved incubating mixtures of these antibodies with tested samples or calibrators in streptavidin-coated plates, followed by subsequent steps to measure fluorescence. The Access BNP assay, utilizing specific MAbs, was also employed for detection of BNP and proBNP.

Samples were collected from patients diagnosed with HF based on symptoms and confirmed through echocardiography and x-ray examination. Samples were processed to obtain plasma or serum and stored appropriately for subsequent analysis. Pooled plasma or serum from healthy individuals served as negative controls. All procedures involving human samples complied with ethical guidelines.

BNP as well as proBNP were extracted from plasma using the Sep-Pak C18 cartridges according to established protocols. The eluates were subsequently dried using a reduced pressure evaporator.

Individual patient plasma samples or extracts were subjected to GF-FPLC using a Superdex 75 column equilibrated with a suitable buffer. Eluted proteins were analyzed, and the column was calibrated using standard proteins. Recombinant and synthetic BNP peptides were included for validation purposes.

Results

This investigation led to the generation of 65 MAbs, whose epitope specificities were determined using synthetic peptides that corresponded to different parts of BNP. Subsequent testing revealed that all MAbs were capable of recognizing both recombinant proBNP and synthetic BNP in direct ELISA assays. Among these, specific interactions with various BNP peptides were observed: three MAbs recognized peptide 1–10, 17 recognized peptide 11–22, 1 recognized peptide 17–23, and 31 recognized peptide 26–32; the epitope specificities of 13 remained undetermined.

Further investigations used BNP-specific MAbs in sandwich-IFA assays with synthetic BNP or HF patient serum. MAbs targeting epitopes 11–22 and 26–32 efficiently detected both endogenous antigen and synthetic BNP. Antibodies 50E126–32 and 24C511–22 had the highest detection limit, aiding in proBNP and derivative analysis in HF patient plasma.

In developing an immunoassay for NT-proBNP quantification, 84 MAbs were chosen for their recognition of recombinant NT-proBNP in ELISA. These MAbs displayed specificity to different regions of NT-proBNP, and epitope mapping was conducted using a library of synthetic peptides. Testing in sandwich-IFA assays revealed that MAbs recognizing remote epitopes could efficiently detect both recombinant proBNP and NT-proBNP, albeit with a lower limit of detection. However, only a few MAb combinations demonstrated reactivity with endogenous antigen from plasma.

Certain MAb pairs, particularly those targeting NT-proBNP regions 13–27 and BNP regions 11–22 or 26–32, showed high sensitivity in detecting endogenous antigen. The 50E1–16F3 pair was most sensitive to both recombinant and endogenous proBNP, with 50E1 targeting BNP's 26–32 site and MAb 16F3 specific to NT-proBNP's peptide 13–20.

The immunoassay validation included typical calibration curves for NT-proBNP, proBNP, and BNP, showing linear ranges and detection limits. Within-assay imprecision was assessed, and recoveries from patient plasma samples were determined. Gel filtration (GF) studies were conducted on plasma samples of patients for the characterization of the molecular forms of proBNP-derived products and also for the establishment of their ratios.

Furthermore, the impacts of preliminary extraction of the proBNP:BNP ratio were investigated, demonstrating significant differences in ratios between non-extracted and extracted samples. Finally, the specificity of the in-house BNP assay was confirmed by comparing it with a commercial assay.

Table 1: Summary of Epitope Specificities of MAbs for BNP Quantification.	
Epitope Range	Number of MAbs
1–10	3
11–22	17
17–23	1
26–32	31
Undetermined	13

Table 2: Summary of Epitope Specificities of MAbs for NT-proBNP Quantification.	
Epitope Range	Number of MAbs
1–24	14
13–27	24
28–45	19
46–60	13
61–76	15

Table 3: Detection Limits and Linearity of Assays.			
Assay	Detection Limit (ng/L)	Linear Range (ng/L)	R^2 Value
BNP	0.4	1–200,000	0.9985
NT-proBNP	10	15–100,000	0.9972
proBNP	3	10–180,000	0.9976

Discussion

In our study, GF method to segregate proBNP and its derivatives in plasma was employed, subsequently subjecting the markers to analysis using three highly sensitive, newly developed, and antigen-specific research IFAs. Our results demonstrate that within the plasma of patients with HF, the immunological reactivity associated with proBNP predominantly corresponds to a single peak representing a peptide having 37 kDa molecular mass. Utilizing the BNP-specific assay, we were able to identify two distinct peaks, with the major peak aligning precisely with the proBNP peak. Conversely, the secondary (minor) peak was observed within the 4–6 kDa range, indicative of BNP presence.

Examining the ratios of proBNP:BNP across HF patient plasma revealed variations in the patients. ProBNP consistently exceeded BNP levels by 1.8- to 10.8-fold in all cases. These findings suggest that proBNP is the primary antigen contributing to the immunological activity detected by the Beckman commercial assay as well as our experimental BNP assay.

For the improvement of assay sensitivity, we created extensive MAb panels targeting NT-proBNP and BNP, spanning their entire sequences. To validate assays, we used HF patient serum and plasma alongside synthetic/recombinant antigens.

This comprehensive approach enabled us to address two critical challenges. Firstly, we successfully identified epitopes that undergo alterations or modifications in endogenous antigens compared to their synthetic or recombinant counterparts. Secondly, we pinpointed MAb combinations capable of recognizing endogenous molecules with equivalent efficacy and sensitivity as their synthetic or recombinant counterparts.

Our research uncovered that MAbs targeting the central region (region 28–60) of the NT-proBNP molecule showed significantly diminished recognition ability towards the antigen in human blood. This is likely due to posttranslational modifications, particularly glycosylation, in the central portion of NT-proBNP, impeding antibody interaction with the endogenous antigen.

Further insights from Schellenberger et al. corroborated our observations, indicating that proBNP expressed in CHO cells exists as a glycoprotein with multiple glycosylation sites predominantly located in the central part of the molecule [14]. Additionally, it was demonstrated that proBNP in the blood of patient undergoes glycosylation. Consequently, the glycosylated central portion of the NT-proBNP molecule exhibit impaired recognition of the endogenous antigen. Thus, we advocate for the utilization of MAbs targeting regions unaffected by glycosylation in NT-proBNP and proBNP assays to ensure accurate and reliable detection.

Chromatography has long been the predominant method for analyzing proBNP, NT-proBNP, and BNP circulating in human blood. However, the immunoassays employed to quantify antigens in chromatography-separated samples often suffered from limited sensitivity. Consequently, it was necessary to concentrate the samples before applying them onto the chromatography column to counteract the dilution effect inherent in the chromatographic process [15, 16].

To ensure the reliability of our findings, we compared a plasma sample using both a commercial BNP assay and our own BNP assay. Before extraction, both assays revealed two peaks: one for proBNP and another for BNP. The proBNP:BNP ratios were 8.6 (commercial) and 10.8 (our own). Preceded by the extraction, the proBNP and BNP peaks became similar in height, resulting in proBNP:BNP ratios of 0.9

(Beckman) and 1.09 (in-house). Our results align with those of previous studies, although slight variations may arise from methodological differences [17].

Conclusion

In conclusion, our study demonstrates the development of highly sensitive immunoassays for the detection as well as quantification of proBNP, NT-proBNP, and BNP in human blood. By utilizing a combination of MAbs and advanced analytical techniques, we were able to accurately characterize the molecular forms of these peptides circulating in plasma from HF patients. Our findings contribute to a better understanding of the pathophysiology of HF and may have implications for the diagnosis and management of this condition in clinical settings.

Funding Source

None

Conflict of Interest

None

Permission

Permission was taken from the committee

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