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Review Article

N-terminal pro-brain natriuretic peptide; current trends in *in-vitro* diagnostics

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ABSTRACT

N-terminal pro-brain natriuretic peptide is the prime standard biomarker used for heart diagnosis and prognosis. Owing to the acute response and considerable half-life, the N-terminal pro-brain natriuretic peptide is the most reliable biomarker to identify a cardiac injury. N-terminal pro-brain natriuretic peptide can act as an independent risk factor for the COVID-19 infected patients, but the exact reason for raise in the level of biomarker is still unclear. Various immunological platforms like immunofluorescence, enzyme linked immunosorbent assay, lateral flow immunoassay, chemiluminescence immunoassay, stable isotope standards capture by anti-peptide antibodies assay are used to detect the N-terminal pro-brain natriuretic peptide from human blood. Chemiluminescence immunoassay lead the *in-vitro* diagnostic platform for the determination of N-terminal pro-brain natriuretic peptide due to high stability, ultra-sensitivity, specificity, and high throughput.

1. Introduction

The thought that heart can also act as an endocrine organ arose with the findings of atrial natriuretic peptides (ANPs) by de Bold et al.,[1] later Sudoh et al.[2] discovered brain natriuretic peptides (BNPs) in 1988. The prohormones such as proANP and proBNP are secreted from cardiac atria and ventricles respectively in response to atrial/ ventricular distention. These precursor hormones then breakdown into biologically active ANP and BNP and inactive amino terminal proANP (NT-proANP) and amino terminal proBNP (NT-proBNP).[3–5] In vitro studies suggested that proteolytic enzyme furin is responsible for the splitting of prohormone into two fragments.[6] The importance of natriuretic peptides in diagnostic field is highlighted with the discovery of ANP and BNP. The enhanced production and release of the natriuretic peptides were observed in heart failure (HF) conditions. In fact, BNP and NT-proBNP have turn out to be the characteristic biomarkers for HF diagnosis and prognosis, excellent than ANP and NT-proANP.[7] According to various studies, BNP and NT-pro BNP in the blood sample can be used as a primary diagnostic tool for acute and chronic heart failure. NT-proBNP is used to monitor medical efficiency of HF drug and to distinguish dyspnea that caused by heart failure from other diseases. The concentration of NT- proBNP is proportional to the level of heart failure.[8–12] In this review, we discuss the recent advances in NT- proBNP as a cardiac marker and potential clinical implications in the diagnostic field.

2. Biochemistry and Physiology of BNPs

The human BNP is encoded by the gene Nppb, which is positioned on chromosome 1 have 1992 base pairs including 3 exons and 2 introns. The gene has a highly conserved area,

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TATTAT.[13] After translation, mRNA is synthesized which encodes a precursor protein of BNP called pre-proBNP having 134 amino acids. The first 26 amino acid sequence of pre-proBNP is known as signal peptide, which undergo sudden degradation to form a prohormone, proBNP having 108 amino acids. The prohormone splits into 32 amino acid sequence of biologically active BNP hormone and 76 amino acid sequence of n-terminal part called NT-proBNP.[3,13] The 108 amino acid proBNP is an O-glycosylated protein in which the NT-proBNP part has 7 glycosylation sites while BNP part is non- glycosylated. The 7 glycosylation sites are at T36, S37, S44, T48, S53, T58, and T71 amino acid residues.[14] The glycosylation status of one amino acid, T71 is important for the proteolytic cleavage of proBNP into BNP and NT- pro BNP. T71 is found close to the cleavage site between the amino acid residues R76 and S77. The cleavage can only occur if T71 is not glycosylated. Majority of the unprocessed proBNP found in circulation has an O-glycan on T71 whereas the same amino acid in NT-proBNP is not glycosylated. The active BNP is forming a ring structure with a disulphide bridge between two cysteine molecules.[3,15,16] During the embryonic and fetal stages, Nppb gene is expressed mostly in the cardiac muscle cells. After birth also the gene continue to express in the heart.[17,18] (Figure 1).

BNP and NT- proBNP, both molecules secreted into blood stream. The key prompt for their over production and release is the cardiac dilation. The activation of BNP takes place when it binds to certain receptors present in the cell surface named natriuretic peptide receptor type A, B and C (NPR-A, NPR-B, NPR-C). The circulating BNP binds to NPR-A and NPR-B triggering intracellular cGMP production. As a result of this, some physiological events occur such as diuresis, natriuresis, peripheral vasodilatation, and inhibition of the renin-angiotensin- aldosterone system and the sympathetic nervous systems.[13,19] The binding of BNP to the NPR-C promote the clearance of BNP from the circulating system. The clearance can also occur through the proteolysis by peptidases especially the neutral endopeptidase (NEP). Most of the BNP degradation takes place in lungs and kidney. Whereas the clearance of NT- proBNP is through renal excretion.[3,13,15,20] Reports concluded that BNP has a half-life of 20 minutes although NT- proBNP is more stable, has a half-life of approximately 120 min., which shows the higher concentration of NT-proBNP in the blood compared to BNP.[3,21–23] (Table 1) Because of this reason, NT- proBNP turn out to be the gold standard for the diagnosis, prognosis, and treatment of heart failure.

3. Clinical Significance of NT- proBNP

A biomarker should possess certain criteria to be clinically relevant. It must be sensitive, specific to the disease, the measurement to be standardised and easy to perform, the level must show clinical and prognostic status. Diagnostic and prognostic value of NT-proBNP and personalized HF treatment based on NT- proBNP have been already established.[8] Currently Roche NT-pro assays are widely used for clinical practices, because of their good diagnostic and prognostic accuracy. The first-generation assay was based on polyclonal antibodies, specific for the regions 1-21 and 39-50 whereas the second-generation uses monoclonal antibodies (mAbs) specific for the central region of NT-proBNP: 22-28 and 42-46.[25] Even Abbott Diagnostics, considered as the pioneer of BNP assays preferred the NT-proBNP as a better option than BNP. EDTA plasma samples are suggested for BNP assay to obtain more stability whereas NT-proBNP values can be evaluated from plasma or serum. The storage stability of NT-proBNP is better compared to BNP with only 10% decrease in actual value after stored at -20 degree Celsius for 2 years whereas BNP has 50% decrease after 2 to 4 months of storage under same conditions.[26,27] Thus, there are many advantages of using NT-proBNP over BNP such as longer half-life, diversity of specimen types, and better storage stability. First line diagnosis of HF with echocardiogram

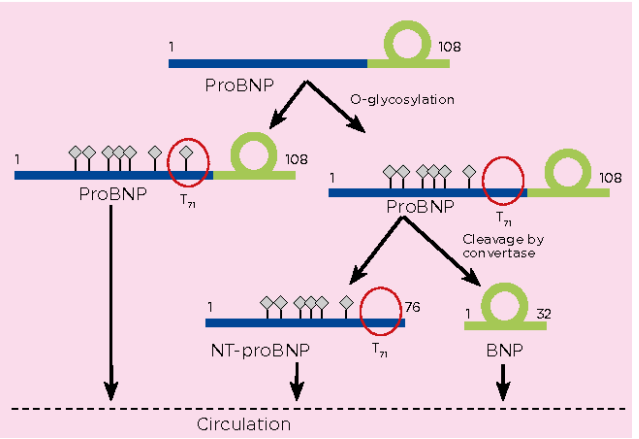

Figure 1: Scheme of proBNP, BNP and NT-proBNP processing. ProBNP is formed after the translation, and it gets glycosylated at different sites. Two sets of proBNP formed with a difference in the T71 glycosylation status: glycosylated at T71 and non-glycosylated at same site. Only non-glycosylated proBNP at T71 undergoes enzymatic cleavage to form NT-proBNP and BNP whereas glycosylated proBNP at T71 remains unprocessed.[24]

Table 1: Comparison of BNP and NT-proBNP

BNP versus NT-proBNP		
	BNP	**NT-proBNP**
Amino acids	32	76
Molecular weight (kDa)	3.5	8.5
Half-life (min)	20	60-120
Hormonal activity	Yes	No
Clearance	Lungs, Kidney	Kidney

(ECG) plus NT-proBNP test giving more accuracy than ECG alone. NT-proBNP concentration may be higher in certain disease conditions such as acute coronary syndrome, older age, renal dysfunction, pulmonary hypertension, pulmonary embolism, sepsis, anaemia, atrial fibrillation, right ventricular dysfunction) and lower in genetic variation, obesity, constrictive pericarditis, flash pulmonary edema or end-stage cardiomyopathy.[28,29]

3.1. Elevated NT-proBNP in COVID-19

The occurrence of coronavirus disease (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been associated with multi-organ dysfunction including respiratory diseases, cardiac injury, and various others. Many studies reported that blood cardiac markers especially cardiac troponin I and NT-proBNP levels increases in accordance with COVID-19. Patients with moderate symptoms have no notable cardiac injury. So, the studies concluded that the level of cardiac markers increases according to the severity of the viral infection. The mechanism of SARS-CoV-2 induced cardiac injury was still unclear. From the result of autopsy, a few interstitial mononuclear inflammatory infiltrates were observed in heart, indicating an inflammation induced cardiac injury. Viral particles may bind at the binding site of angiotensin converting enzyme related carboxypeptidase (ACE2). This infection prevents the proper supply of oxygen to the cardiac muscle cells which may stimulate the elevation of cardiac markers like NT-proBNP, finally leads to the cardiac injury. Another possibility is the binding of SARS-CoV-2 to the ACE2 receptor causing an uncontrolled production of angiotensin 2 (AGN II) and limited the synthesis of angiotensin 1-7. This will cause an anti-inflammatory effect, which leads to the secretion of NT-proBNP.[30–33]

4. Analytical Tools

BNP and NT-proBNP have been assessed as cardiac markers and both are based on non-competitive (sandwich) immunoassay using capture and detector antibodies.[29] There are variety of analytical methods in clinical and diagnostic field, specific for natriuretic peptides including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunofluorescence assay (IFA), microfluidics, recently developed electrochemiluminescence immunoassay (ECLIA) and so on. Contemporary methods for the determination of circulating NT-proBNP are based on the sandwich immunoassay. The target antigen is bound between capture and detector antibodies to form a sandwich. NT-proBNP has been measured using various diagnostic platforms such as Roche diagnostics Elecys, Siemens Immulite, Dade systems, Ortho Vitros ECi etc.[34] We briefly discuss most important and recent advances in analytical techniques used to measure the circulating NT-proBNP level.

4.1. Immunofluorescence assay

The development of immunofluorescence assays provides the detection of NT- proBNP in 15- 20 minutes. This simple and rapid method widely used in clinical diagnosis, ensures a primary care for heart failure. The basic principle of IFA is founded on sandwich immunoassay. Here one monoclonal antibody immobilized on a nitrocellulose membrane (test line) and another monoclonal antibody is conjugated with fluorescent protein. After the addition of sample (serum, plasma, or whole blood), the analytes were captured by fluorescently labelled antibody and forms antigen- antibody complex. This complex moves towards the test line by capillary action and captured by the antibody coated there to form sandwich complex. Fluorescence was measured by analyzer and the concentration of analytes could be calculated. The florescence intensity is directly proportional to the analyte concentration.[35]

4.2. Enzyme-linked immunosorbent assay

ELISA offers a quantitative measurement of NT- proBNP. In ELISA, antibodies are linked to an enzyme specific to a reaction of a substrate. Alkaline phosphatase (ALP), horse radish peroxidase (HRP) and β-galactosidase are the commonly used enzymes. When these antibodies bind to the NT-proBNP, the substrate is added to it, and a catalysed reaction produces a colour change that helps quantify NT-proBNP. The quality and quantity of the assay depends on the antigen-antibody interaction. The enzyme-substrate reaction completes within 30-60 min and stops by adding sulfuric acid. A microtiter plate reader is used to detect coloured reaction.[36,37] ELISA has several limitations including the necessity of a large sample volume, long detection time of about four hours, and the cost of instruments. Also compared to other types of assays, ELISA is not sensitive enough to detect too small NT-proBNP levels.[38]

4.3. Chemiluminescence immunoassay

Chemiluminescence immunoassay is a modern and versatile immunoassay has obtained remarkable attention and been utilized for the quantitative determination of protein analytes because of its high stability, ultra-sensitivity, and specificity. Chemiluminescence is defined as the emission of light because of chemical reaction. The methods can be direct or indirect by using luminophore markers and enzyme markers respectively. Acridinium and ruthenium esters are the commonly used luminophores whereas enzymatic markers used in indirect method include alkaline phosphatase with adamantyl 1,2-dioxetane aryl phosphate (AMPPD) substrate and horseradish peroxidase with luminol or its derivatives as substrate.[39]

Roche Diagnostics has a variety of immunoassay analysers like Elecsys 1010, 2010 and E170 to perform NT-proBNP assay. Roche uses two polyclonal antibodies designed at epitopes on residues 1-21 and 39- 50, labelled with biotin and ruthenium complex respectively. Both binds to NT-proBNP to form a sandwich. Detection is by the addition of streptavidin labelled microparticles. The immune complex is bound through biotin-streptavidin interaction. The assay will take only 18 min, has a reference range of 5-35,000 ng/L.[40]

4.4. Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) assay

SISCAPA is an extended version of stable isotope dilution used for the quantification of analytes by mass spectrometry. Instead of measuring the whole analyte directly SISCAPA utilizes proteolysis to degrade protein samples into smaller units. SISCAPA is an immuno-mass spectrometric method in which a high affinity antibody specific for a specific peptide is used to enhance the target analyte from a complex mixture such as human plasma or serum digest, elute and quantify analyte against a known amount of a stable isotope internal standard. The system utilizes an 'addition-only' protocol whereby liquid reagents are added to the samples throughout the procedure without any additional steps like centrifugation, long chromatographic separation etc.[41,42]

Studies reported that NT-proBNP can also be quantified using SISCAPA technology. Magnetic beads coated with anti-peptide antibodies bind specific target peptides from enzymatic digests of samples such as plasma and serum, after which the beads are washed to remove unbound particles, and the bound, purified peptides are eluted in small volumes for injection into a mass spectrometric system.[43]

4.5. Other methods

The application of optical labels in immunoassay can be used to detect the amount NT-proBNP in the specimen. According to Goryacheva et al.[44] blood sample is taken onto a disposable plastic cartridge, inside that blood cells are trapped within a semipermeable membrane. NT-proBNP containing plasma move into a microfluidic system and is transported to the reaction chambers by capillary forces. NT-proBNP binds to the functionalized with anti-NT-proBNP antibody imaging surface. The detection antibody along with the dried magnetic particles dissolved within the plasma, move to imaging surface by applying a magnetic field and bind to the complex of NT-proBNP molecule and anti-NT-proBNP-antibody. In the final step, unbound magnetic particles are washed off from the imaging surface using a magnetic field gradient in the opposite direction. Specifically bound magnetic particles on the imaging surface can be optically detected using Fourier-transform infrared spectroscopy (FTIR).

Franziska et al.[45] came up with an idea of using dry-reagent microfluidic biosensor for the detection of NT-proBNP with the help of silver nanoparticles. It has a simple detection approach, required very less sample volume as finger pricked (less than 10μl), also stability of the reagent is good because of its dried form. Here, silver nanoparticles are dried inside the microfluidic channel in a matrix of trehalose sugar fixed with sodium sulphite as oxygen scavenger. This effectively prevented the oxidation of silver nanoparticle and facilitated dry and ready-to-use storage for minimum 18 weeks. Based on this, laser-cut flow chips were developed containing all bioassay reagents needed in a ready-to-go dry format.

5. Discussion

5.1. NT-proBNP as a cardiac biomarker

Heart failure is a public health issue shows high mortality and morbidity rates. In order to tackle this problem, it is necessary to develop and adopt new techniques in diagnostic field. NT-proBNP is considered as one of the best cardiac markers in this field. NT-proBNP is a protein hormone that originates from heart and blood vessels. Immunoassays measure the amount of these hormone in blood sample. It is normal to have certain amount of hormone in the blood, but greater than normal levels may be a sign of heart failure. High level of NT-proBNP also present in some conditions such as kidney failure, sepsis, lung disorders etc. The half-life of this hormone is around 120 min.

5.2. Immunoassays

Currently, various immunoassays with different principles are available commercially to determine the level of NT-proBNP. Most of the immunoassays are based on non-competitive or sandwich method using a capture antibody and detector antibody. Several diagnostic platforms are provided by multinational health care companies such as Roche diagnostics, Siemens, Abbott laboratories etc. NT-proBNP can be quantified using different techniques including immunofluorescence assay, ELISA, CLIA, SISCAPA, microfluids methods and so on. Different methods have different sample volume, time of detection, sample type, accuracy, specificity, and throughput. Immunofluorescence assays provide simple and rapid detection where antibodies are immobilized on a nitrocellulose membrane. ELISA is a time taking procedure and need large sample volume. CLIA is modern and highly advanced technique and provide high throughput results. Here luminescence is produced as result of chemical reaction. There are other techniques like SISCAPA and assays based on microfluids are also there in the market

6. Conclusion

Chemiluminescence immunoassay is the best among all available immunoassays in the determination of cardiac biomarkers. Because of its high stability, ultra-sensitivity, specificity, and high throughput CLIA is considered as the best diagnostic platform for the determination of NT-proBNP.

7. Conflict of Interest

None.

8. Source of Funding

None.

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