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NT-proBNP stability assessed by several sandwich immunoassays utilizing monoclonal antibodies with different epitope specificity.

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INTRODUCTION

NT-proBNP is a useful marker for heart failure, including congestive heart failure. NT-proBNP measurements are becoming routine in clinical practice. But still reports on long term in vitro stability of NT-proBNP molecule are conflicting. Conflicting data could be explained (at least partially) by different epitope specificity of the antibodies utilized in different immunoassays, which are used to assess the stability of the antigen in the sample. The aim of the current study was to reveal the most stable parts of NT-proBNP molecule and epitope specificity of monoclonal antibodies (Mabs). These Mabs could be used for the development of sandwich immunoassay less sensitive to the proteolytic degradation or other modifications of NT-proBNP molecule, which could happen during the long-term storage of blood samples at +6 ºC and at room temperature (+22 ºC).

Materials and Methods

Monoclonal antibodies and antigen: In this study we used monoclonal antibodies (HyTest, Finland) specific to different fragments of human NT-proBNP molecule. Mab 5B6 recognizes amino acid residues (a.a.r.) 1-12 (most likely the very N – terminal part of the molecule), Mab 29D12 – a.a.r. 5-12, Mabs 13G12, 15F11, 18H5 and 7B5 – a.a.r. 13-27, Mabs 5E2 and 16E6 – a.a.r. 28-45 and Mabs 15C4, 21E6 and 24E11 – a.a.r. 61-76 (Fig. 1).

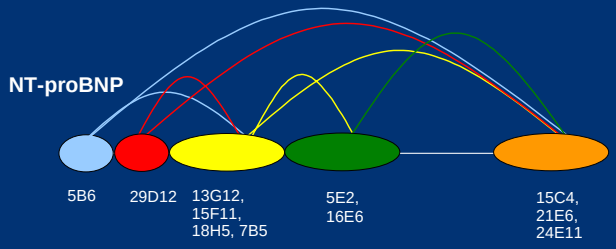

Figure 1: Epitope location of Mabs used in ten sandwich immunoassays.

Serum samples: Serum samples from 39 cardiac failure patients were pooled and incubated (with of 0.1 % sodium azide added as a preservative) for 0, 1, 3, 8, 24, 48 and 72 hours at +6 ºC and at room temperature. After incubation samples were frozen and stored at – 70 ºC until immunological activity was measured.

Sandwich time-resolved immunofluorometric assay: NT-proBNP immunological activity of the samples was assessed in 10 sandwich assays utilizing two-site combinations of Mabs specific to different parts of NT-proBNP molecule. Detection Mabs were labeled with TEKES – stable europium chelate. During the first step plates were incubated with coating Mabs for 30 minutes at room temperature. Unbound antibodies were washed away and then detection antibodies and antigen-containing samples were added and incubated for 30 minutes at room temperature. After washing and addition of enhancement solution fluorescence was measured with Victor 1420 Multilabel Counter (Wallac-PerkinElmer, Finland).

Results and Discussion

The lowest stability – 63-77% and 8-16% of the initial immunological activity after 72 hours of incubation at +6 ºC and at room temperature, respectively – was demonstrated for the assays utilizing Mab 5B6, specific to the very N-terminal part of the molecule (Fig. 2). Significant decrease in immunological activity measured by Mab pairs 5B6-13G12 and 5B6-24E11 (Fig. 3) was observed already after 24 hours of incubation. However in assays utilizing Mab 29D12 with the epitope located more distantly from the N-terminal end of the molecule (peptide 5-12) NT-proBNP demonstrated significantly better stability.

Figure 2: Immunological activity in pooled human serum after 72 hours of incubation at +6º C (A) and +22 ° C (B).

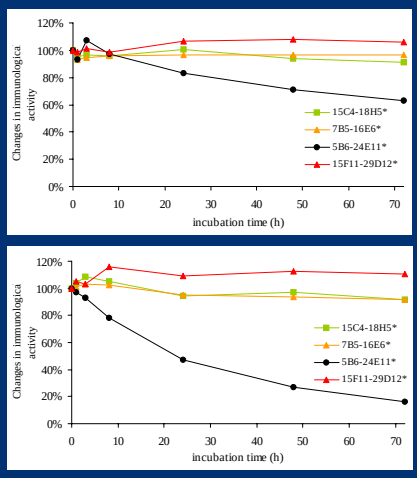

Figure 3: Changes in immunological activity during 72 hours of incubation at +6 ºC (A) and at + 22 °C (B).

Assays with the antibodies specific to the C-terminal part (peptide 61-76) of NT-proBNP had significantly better stability (80-100% and 60-90% after 72 hours of incubation at +6 °C and at room temperature, respectively). The stability of the antigen assessed by the assays with antibodies specific to the central part of the molecule was even greater - for some Mab combinations slightly growing in time. We observed the highest stability (106% and 110%) in the assay utilizing capture Mab 15F11 specific to peptide 13-27 and detection Mab 29D12 specific to peptide 5-12.

The dependence of immunological activity on Mab epitope specificity is represented in Figure 4. Immunological activity was compared in four assays, each utilizing one Mab specific to stable central part of the molecule (peptide 13-27) and another Mab specific to one out of three other peptides; 1-12, 5-12, 28-45 and 61-76. We observed significant decrease of activity only in assay utilizing Mab 5B6 (the very N-terminal part of the molecule). In other assays NT-proBNP demonstrates apparently high stability even after 72 hours of incubation at room temperature. Thus, for utilization in the assays, antibodies specific to the very N – terminal part of the molecule should be avoided because of low stability of the analyte fragment recognized by such antibodies.

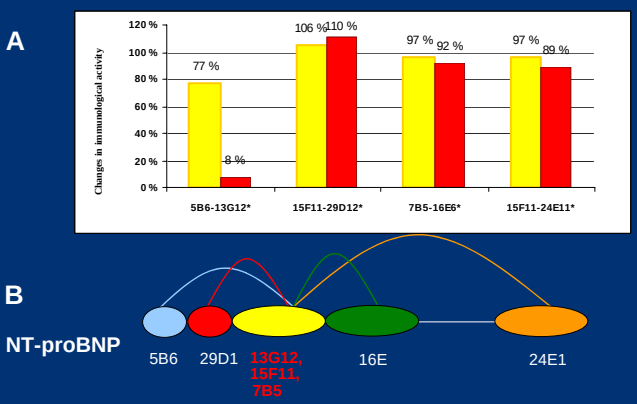

Figure 4: (A) BNP immunological activity in pooled human serum after 72 hours of incubation at +6 ºC (yellow columns) and +22 ºC (red columns). (B) Epitope location of Mabs used in immunoassays

Conclusions:

- **The apparent stability of NT-proBNP significantly depends on the specificity of the antibodies utilized in the assay.**
- **In the assay utilizing antibodies specific to the peptides 13-27 (Mab 15F11) and 5-12 (Mab 29D12) endogenous NT-proBNP reveals the highest stability.**