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# N-glycosylated IGFBP-4 could be less susceptible to PAPP-A mediated proteolysis than the non-glycosylated form

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## Introduction

N- and C-terminal proteolytic fragments of IGFBP-4 (NT-IGFBP-4 and CT-IGFBP-4) are strong predictors of major adverse cardiac events in patients presented with myocardial ischemia and patients with type 1 diabetes [1, 2].
NT-IGFBP-4 and CT-IGFBP-4 are the products of PAPP-A dependent cleavage of full-length IGFBP-4. PAPP-A specifically cleaves IGFBP-4 between Met135 and Lys136. We have previously developed sandwich immunoassays based on monoclonal antibodies (mAbs) that are specific to proteolytic neo-epitopes on NT-IGFBP-4 and CT-IGFBP-4 and have less than 1% of cross-reactivity to the full-length IGFBP-4 [1].
It has been shown previously that a fraction of circulating IGFBP-4 contains glycosylated Asn104 that is located in the N-terminal part of protein. Asn104 is relatively close to the PAPP-A-specific cleavage site and to the epitope of the mAb utilized in NT-IGFBP-4 assay. Thus it can be supposed that measurements of some portion of NT-IGFBP-4 may be compromised by the glycosylation.
Therefore, the aims of the study were:

a) **To evaluate the influence of glycosylation on the immunodetection of NT-IGFBP-4;**
b) **To compare the susceptibilities of glycosylated and non-glycosylated IGFBP-4 to PAPP-A dependent proteolysis.**

## Methods

Affinity chromatography and concanavalin A chromatography were used for the extraction of glycosylated IGFBP-4 and NT-IGFBP-4 from acute coronary syndrome (ACS) patients' plasma. Purified proteins were analyzed using enhanced chemi-luminescence Western blotting (WB) and sandwich HRP-immunoassays based on mAbs combinations IBP185-IBP180 and IBP180-IBP3, respectively (Fig. 1).

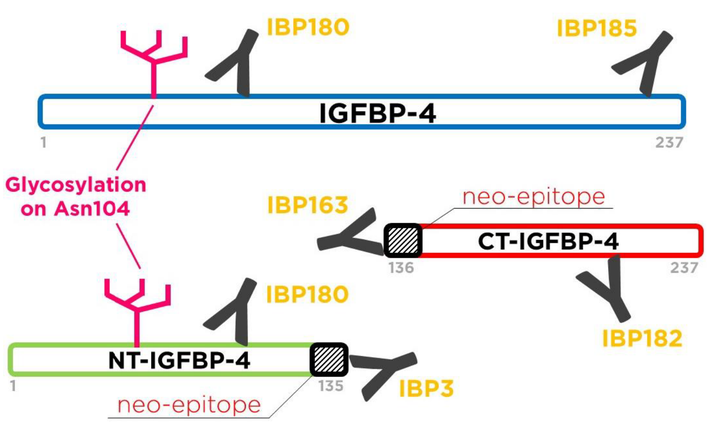


**FIGURE 1. Schematic representation of the IGFBP-4 and its proteolytic fragments' specific sandwich immunoassays based on mAbs to different epitopes of the molecules.**

The composition of the IGFBP-4 glycan was studied using deglycosylases PNGase F and 2-3,6,8-neuraminidase with consequent urea electrophoresis and WB.
To investigate the glycosylation level total fractions of IGFBP-4 and NT-IGFBP-4 were immunoprecipitated from twelve EDTA plasma samples of ACS patients and then analyzed using WB.
To study the influence of IGFBP-4 glycosylation on its PAPP-A dependent proteolysis, the increases in the concentrations of the proteolytic fragments of glycosylated and non-glycosylated IGFBP-4 during the incubation with recombinant PAPP-A were measured. Sandwich fluoro-immunoassays IBP180-IBP3$^{EU}$ and IBP182-IBP163$^{EU}$ were used for NT-IGFBP-4 and CT-IGFBP-4 measurement, respectively (Fig. 1).

**REFERENCES**

1. A.B. Postnikov, T.I. Smolyanova, A. V Kharitonov, D. V Serebryanaya, S. V Kozlovsky, Y.A. Tryshina, et al., N-terminal and C-terminal fragments of IGFBP-4 as novel biomarkers for short-term risk assessment of major adverse cardiac events in patients presenting with ischemia., Clin. Biochem. 45 (2012) 519–24.
2. R. Hjortebjerg, L. Tarnow, A. Jorsal, H.-H. Parving, P. Rossing, M. Bjerre, et al., IGFBP-4 Fragments as Markers of Cardiovascular Mortality in Type 1 Diabetes Patients With and Without Nephropathy., J. Clin. Endocrinol. Metab. 100 (2015) 3032–40.

## Results

**Extraction of glycosylated and non-glycosylated IGFBP-4 and NT-IGFBP-4 from ACS plasma.**
The WB analysis of the proteins extracted from ACS plasma (Fig. 2) revealed the bands corresponding to 25-kDa non-glycosylated IGFBP-4 (lane 2) and 15-kDa NT-IGFBP-4 (lane 4). The glycosylated IGFBP-4 (30 kDa, lanes 1 and 3) and NT-IGFBP-4 (18 kDa, lane 3) was also detected.

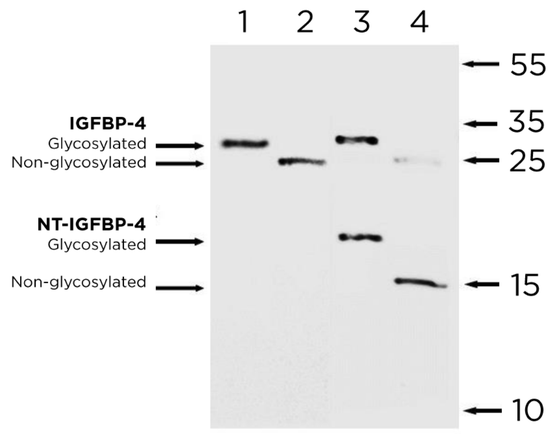


**FIGURE 2. WB of glycosylated and non-glycosylated IGFBP-4 and NT-IGFBP-4 extracted from ACS plasma.** WB, staining with mAb IBP180. Lanes: 1 – glycosylated IGFBP-4; 2 – non-glycosylated IGFBP-4; 3 – preparation of glycosylated NT-IGFBP-4; 4 – preparation of non-glycosylated NT-IGFBP-4.

**Investigation of the IGFBP-4 glycosylation type.**
The presence of circulating glycosylated NT-IGFBP-4 (17260-Da peak; accuracy of mass measurements <0.05%) was confirmed by MS study (Fig. 3).
Glycosylated IGFBP-4 and NT-IGFBP-4 are processed by PNGase F (Fig. 4, A) which cuts N-glycans only. Treatment of NT-IGFBP-4 by 2-3,6,8-neuraminidase leads to the changing of its isoelectric point (Fig. 4, B) suggesting that NT-IGFBP-4 molecule contains sialic acids in the glycan.

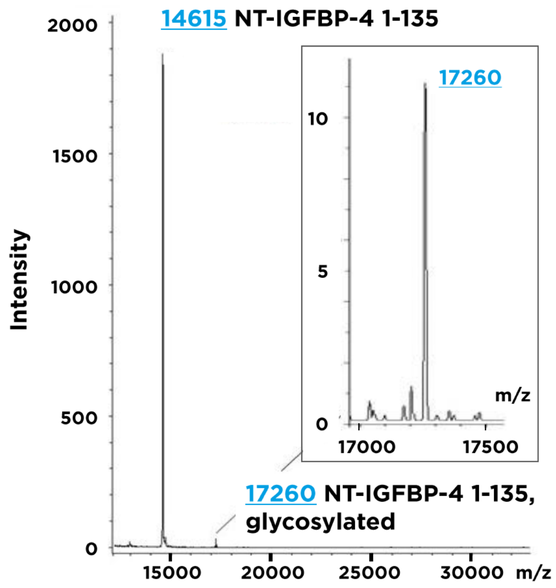


**FIGURE 3. Mass spectrum of glycosylated and non-glycosylated NT-IGFBP-4.** Peak 14615 m/z corresponds to N-terminal fragment 1-135 of IGFBP-4. Peak 17260 m/z corresponds to NT-IGFBP-4 1-135 with N-glycosylation.

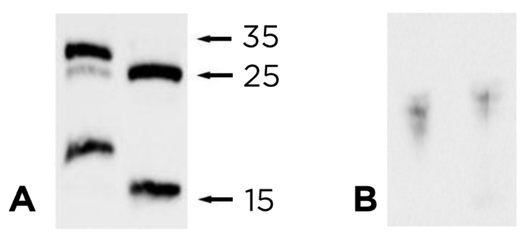


**FIGURE 4. Deglycosylation of IGFBP-4 and NT-IGFBP-4 extracted from ACS plasma with PNGase F and 2-3,6,8-neuraminidase.** WB, staining with mAb IBP180.

**Immunodetection of glycosylated NT-IGFBP-4.**
The glycosylated Asn104 is located in the close vicinity to the epitope of MAb IBP180. Thus the glycosylation can influence the immunodetection of NT-IGFBP-4.
We found that the immunodetection of NT-IGFBP-4 in sandwich HRP-immunoassay IBP180-IBP3 is not affected by NT-IGFBP-4 glycosylation. The immunoreactivities of glycosylated and non-glycosylated NT-IGFBP-4 differs by less than 7.5% (Fig. 5).

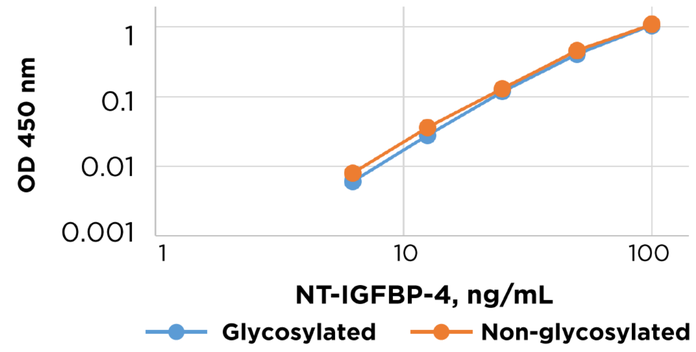


**FIGURE 5. Immunodetection of glycosylated and non-glycosylated NT-IGFBP-4 using sandwich immunoassay IBP180-IBP3$^{HRP}$.**

**Analysis of portions of glycosylated IGFBP-4 and NT-IGFBP-4 in individual ACS plasma.**
To measure the proportion of glycosylated and non-glycosylated IGFBP-4 and to verify the presence of glycosylated NT-IGFBP-4 in the individual plasma of ACS patients the WB analysis of extracted IGFBP-4 forms was performed (Fig. 6).
The portion of glycosylated NT-IGFBP-4 in total NT-IGFBP-4 (9.8-23.5%, mean 15.6%) was lower than the portion of glycosylated IGFBP-4 in total IGFBP-4 (47.2-61.7%, mean 54.8%).

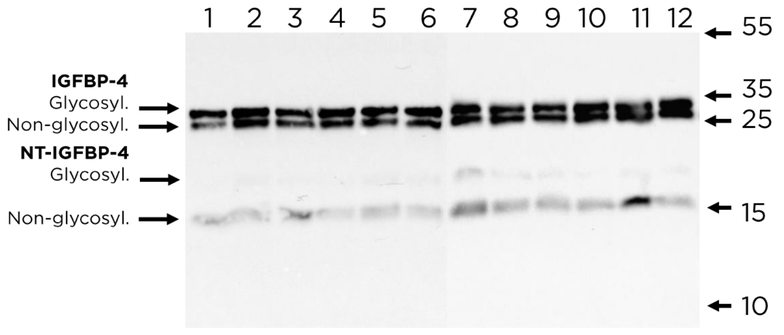


**FIGURE 6. WB of IGFBP-4 and NT-IGFBP-4 stained with mAb IBP180.** Lanes 1-12: twelve individual preparations of total IGFBP-4 and NT-IGFBP-4 extracted from ACS plasma.

**Influence of glycosylation on PAPP-A-dependent cleavage of IGFBP-4.**
To test if glycosylation of IGFBP-4 could affect its PAPP-A dependent proteolysis enzymatically active PAPP-A was incubated with glycosylated and non-glycosylated IGFBP-4 preparations.
Glycosylated IGFBP-4 and non-glycosylated IGFBP-4 demonstrated different rates of PAPP-A dependent cleavage (Fig. 7). The formation of proteolytic fragments derived from non-glycosylated IGFBP-4 was 3-4-fold faster than from glycosylated IGFBP-4.
This observation suggests that the reduced portion of glycosylated NT-IGFBP-4 in the individual ACS plasma is associated with the inhibition of PAPP-A dependent proteolysis of glycosylated IGFBP-4.

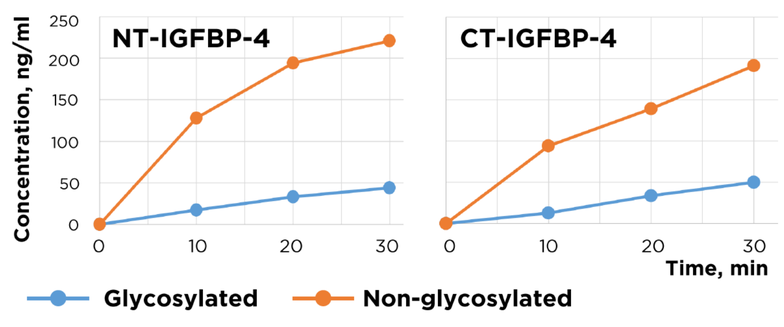


**FIGURE 7. PAPP-A dependent proteolysis of purified glycosylated and non-glycosylated IGFBP-4 preparations.** The of NT-IGFBP-4 and CT-IGFBP-4 were measured using sandwich fluoro-immunoassays IBP180-IBP3$^{EU}$ and IBP182-IBP163$^{EU}$, respectively.

## Conclusions

1. **For the first time, the presence of glycosylated NT-IGFBP-4 in human plasma was shown. The ratio of glyco/total NT-IGFBP-4 in plasma is significantly lower than the ratio of glyco/total full-length IGFBP-4 (9.8-23.5% vs 47.2-61.7%).**
2. **PAPP-A dependent proteolysis of glycosylated IGFBP-4 is 3-4 times less efficient if compared with the proteolysis of non-glycosylated IGFBP-4.**
3. **The glycosylated NT-IGFBP-4 displays the same immunoreactivity as non-glycosylated NT-IGFBP-4 in the fragment-specific assay IBP180-IBP3$^{HRP}$. This immunoassay can be used for the reliable measurement of total (glycosylated and non-glycosylated) NT-IGFBP-4 in the patients' blood.**

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