Insulin-like growth factor binding-protein-4 (IGFBP-4) fragments

Fragments of the insulin-like growth factor binding protein 4 (IGFBP-4) are recognized as being promising new biomarkers of major adverse cardiovascular events risk in patients with acute coronary syndrome (ACS). They are formed from IGFBP-4 by cleavage with pregnancy-associated plasma protein-A (PAPP-A).

IGFBP-4 fragments and PAPP-A

Previously, PAPP-A was suggested as being a potential marker of cardiovascular diseases. Several studies showed that the expression of PAPP-A was significantly increased, especially in unstable atherosclerotic plaques. Furthermore, it was shown that high levels of PAPP-A in circulation could indicate an increased risk of plaque rupture, which, in turn, could lead to a major adverse cardiac event (MACE). Unfortunately, PAPP-A assays have been shown to also recognize PAPP-A in complexes with proMBP, which is not related to atherosclerotic plaques. In addition, PAPP-A measurements have been shown to be influenced by heparin, which is an anti-coagulation agent that is routinely used in the treatment of patients with acute myocardial infarction. All in all, these limitations make PAPP-A an unreliable marker for the monitoring of plaque rupture.

PAPP-A is a metalloproteinase and its main substrate is IGFBP-4. PAPP-A specifically cleaves IGFBP-4 between Met135-Lys136 into two fragments: N-terminal IGFBP-4 (NT-IGFPB-4) and C-terminal IGFBP-4 (CT-IGFBP-4) (see Figure 1). Due to the challenges presented by PAPP-A measurements, our researchers hypothesized that IGFBP-4 fragments could perhaps be used as surrogate markers of enzymatically active PAPP-A.

IGFBP-4 fragments as diagnostic markers

Recent studies have indicated that IGFBP-4 fragments can predict adverse cardiac events in acute coronary syndrome patients (1). Moreover, immunoassays for the fragments are not affected by heparin, such as the PAPP-A assays (2) which would make them more suitable for use with samples from patients who have received heparin as part of their treatment.

Reagents for immunoassay development

At Hytest, we provide monoclonal antibodies (MAbs) that enable the development of quantitative immunoassays for the detection of NT-IGFBP-4 and CT-IGFBP-4 separately (1).

In addition, we provide N-terminal and C-terminal fragments of IGFBP-4 as recombinant proteins.

CURRENT CONCEPT OF CLINICAL UTILITY

Circulating IGFBP-4 fragments could be used as strong predictors of

- Short-term to medium-term cardiac events and death in patients with suspected acute coronary syndrome
- Long-term cardiac death in patients with Type 1 Diabetes
- Short-term to long-term mortality in patients following acute myocardial infarction
- Short-term to medium-term all-cause mortality in patients with acute heart failure

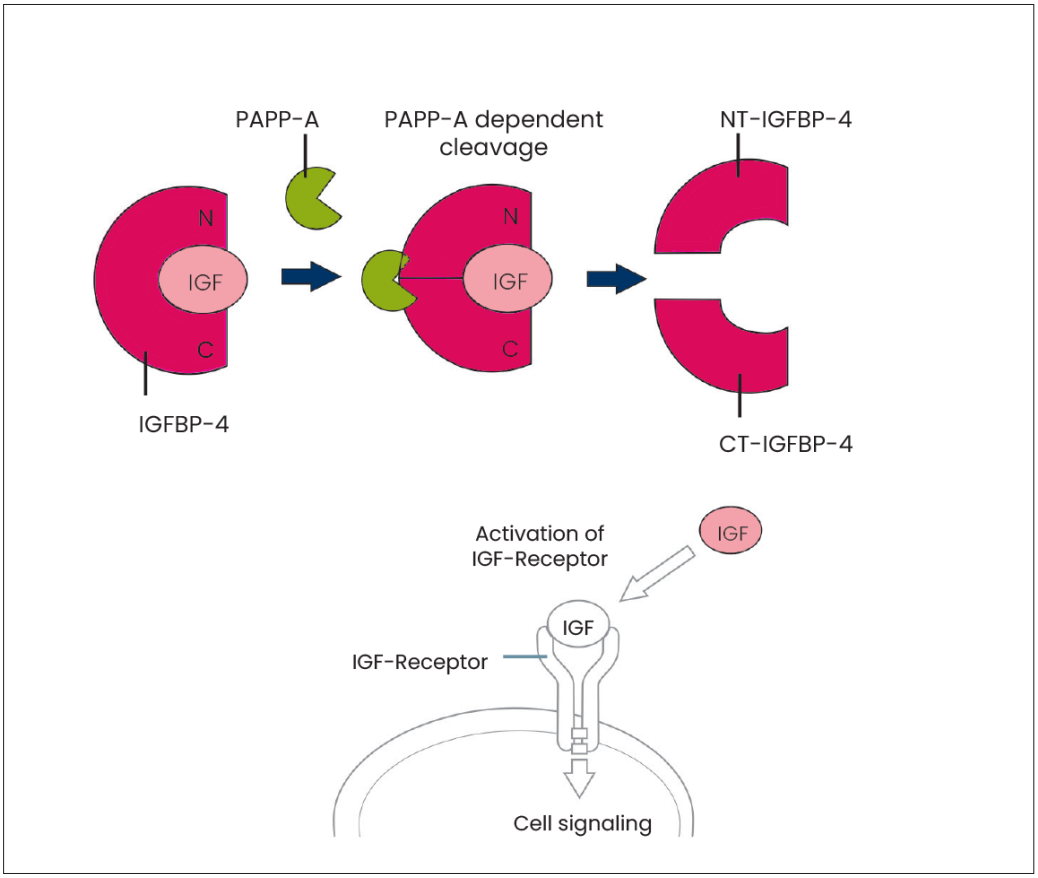

Figure 1.
Scheme of IGFBP-4 cleavage by PAPP-A. *PAPP-A specifically cleaves IGFBP-4 between Met135-Lys136 leading to the formation of two proteolytic fragments, NT- and CT-IGFBP-4. At the same time, IGF is realeased.*

ATHEROSCLEROSIS

Atherosclerosis is a slowly progressing disease in which mainly lipids and inflammatory cells accumulate in the artery walls to form plaques. The formation of plaques can start early in life but they can remain asymptomatic for decades. Plaques narrow the blood vein and at a certain point will start to affect blood flow. This can cause symptoms such as chest pain.

Atherosclerotic plaques are divided into stable and unstable or vulnerable plaques. The latter are prone to rupturing, which is an event that can lead to an abrupt thrombotic occlusion.

Plaque destabilization is a highly complex and multi-factorial process. Several studies have shown that the expression of dPAPP-A is significantly increased in unstable atherosclerotic plaques.

Current diagnosing methods detect coronary stenosis accurately in severe cases but they are not able to reveal the underlying processes. Therefore, there is a need for more accurate risk markers.

IGFBP-4 fragments could perhaps be used as surrogate markers of enzymatically active PAPP-A.

MONOCLONAL ANTIBODIES SPECIFIC TO NT-IGFBP-4 AND CT-IGFBP-4

We have developed two unique MAbs that are specific to the novel epitopes formed when IGFBP-4 is proteolytically cleaved by PAPP-A (1). When combined with MAbs that bind closer to the ends of the full-length IGFBP-4 molecule, it is possible to develop two separate assays that only detect NT-IGFBP4 or CT-IGFBP4 (see Figure 2).

Development of sandwich immunoassays

NT-IGFBP-4 fluoroimmunoassay. The MAb pair IBP3-IBP180 can be used for the detection of the N-terminal fragment of IGFBP-4. IBP3 only recognizes the cleaved fragment while the MAb IBP180 recognizes both forms. A calibration curve for the recombinant NT-IGFBP-4 is shown in Figure 3 A.

CT-IGFBP-4 fluoroimmunoassay. We used the MAb IBP182 as the capture antibody and IBP163 as the detection antibody. The MAb IBP182 recognizes both the full-length IGFBP-4 and the CT-IGFBP-4 fragment, while IBP163 only binds to the epitope formed after cleavage of the full-length IGFBP-4. A calibration curve for the recombinant CT-IGFBP-4 is shown in Figure 3 B.

IGFBP-4 fragments ELISA with HRP-labeled antibodies. The same antibody pairs work well in HRP format; however, the opposite orientations are preferable: IBP163–IBP182HRP for the CT-IGFBP-4 assay and IBP180–IBP3HRP for the NT-IGFBP-4 assay.

Developed IGFBP-4 fragments ELISA assays have shown low cross-reactivity with the full-length IGFBP-4 molecule (<2%) and sensitivity that is suitable for the measurement of IGFBP-4 fragments in the general population. The validation of NT-IGFBP-4 and CT-IGFBP-4 fluoroimmunoassays and ELISA have been described in several publications (1-6).

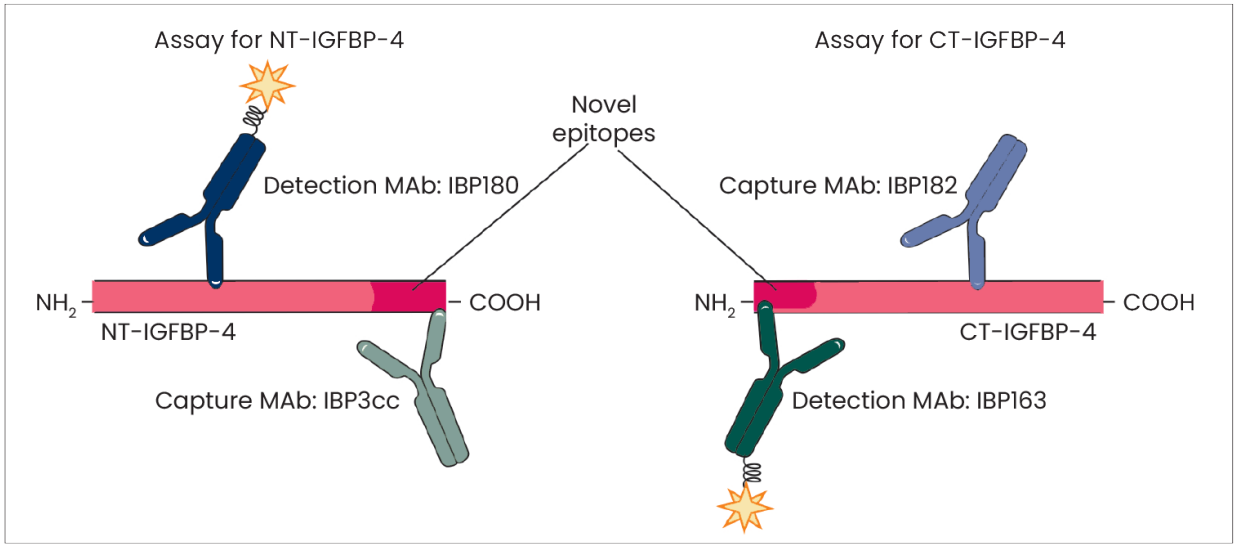

Figure 2.

Schematic representation of fluoroimmunoassays designed to detect only NT-IGFBP-4 (left) and CT-IGFBP-4 (right). *The MAb IBP3cc is specific to the novel epitope of NT-IGFBP-4 and the MAb IBP163 recognizes the novel epitope of CT-IGFBP-4.*

RECOMBINANT ANTIGENS

Hytest provides the unique N-terminal and C-terminal fragments of IGFBP-4 as purified recombinant proteins. These proteins are produced in a mammalian cell line expression system and they do not contain any tags. These recombinant proteins can be used for example as immunoassay standards or calibrators, or as mass standards various applications.

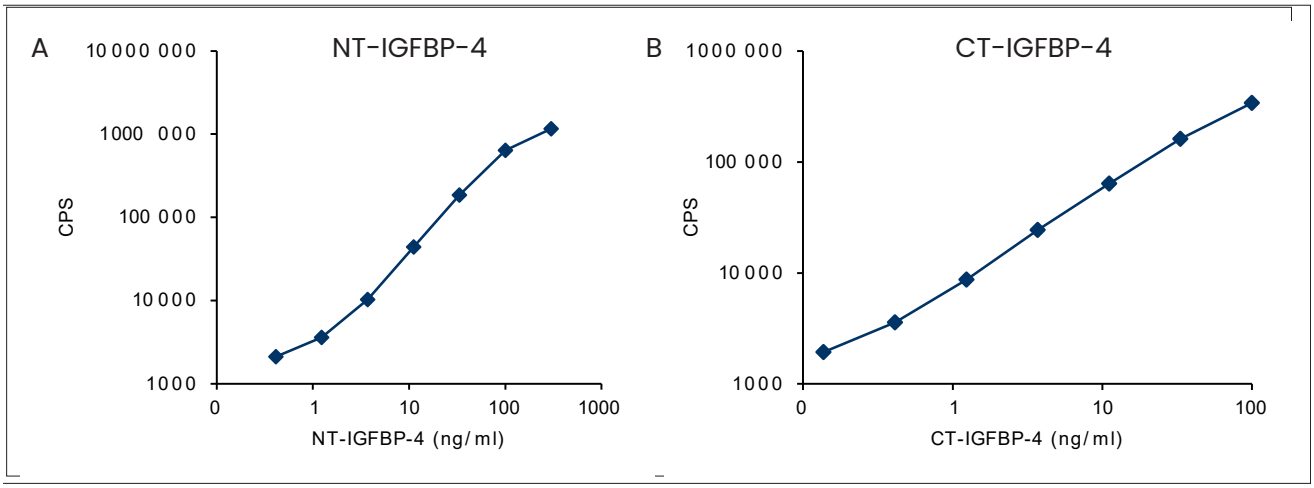

Figure 3.

Calibration curves for (A) NT-IGFBP-4 and (B) CT-IGFBP-4 using sandwich fluoroimmunoassays. *MAb pair IBP3–IBP180 (capture-detection) was used in the NT-IGFBP-4 assay and MAb pair IBP182–IBP163 in the CT-IGFBP-4 assay.*

CLINICAL STUDIES

The clinical utility of IGFBP-4 fragments has been investigated in several studies. We have described below the key clinical studies that have been conducted in order to investigate the use of NT-IGFBP-4 and CT-IGFBP-4 as biomarkers.

I. Postnikov et al., 2012: Prediction of short-term to medium-term cardiac events and death in patients with suspected acute coronary syndrome

In our article (1), IGFBP-4 fragments were measured in the EDTA-plasma samples of 180 patients with suspected non-ST elevation ACS. The diagnosis of non-ST-elevation myocardial infarction (non-STEMI) was based on the presence of signs or symptoms of myocardial ischemia.

Both NT-IGFBP-4 and CT-IGFBP-4 were shown to be strong predictors of MACE including MI and cardiac death in a 6 month follow-up period (the values of area under the ROC curve were 0.86 and 0.81 respectively, see Figure 4; own data not published in the paper).

Kaplan–Meier survival curves for MACE indicated that NT-IGFBP-4 and CT-IGFBP-4 were associated with an increased risk of future MACE: adjusted hazard ratio 13.79 and 7.93 respectively. It should be noted that the majority of end points occurred within the first 3 months.

In this article we also described the development of the MAbs and novel immunoassays for detecting IGFBP-4 fragments.

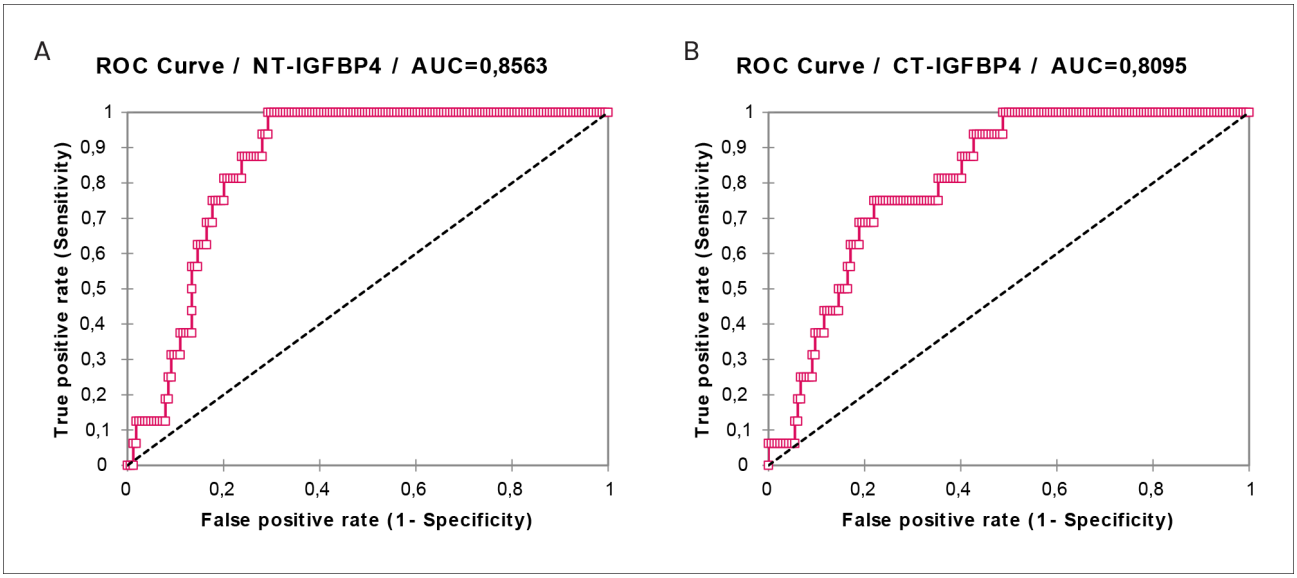

Figure 4.

ROC curve analysis of MACE prediction at 6-months on the basis of NT-IGFBP-4 (A) and CT-IGFBP-4 (B) measurements. *For the detection of NT-IGFBP-4 (A), a sandwich immunoassay utilizing antibodies IBP3 (capture) and IBP144 (detection) was used. CT-IGFBP-4 (B) was detected using antibody pair IBP182-IBP163 (capture-detection).*

II. Hjortebjerg et al., 2015: Prediction of long-term cardiac death in patients with Type 1 Diabetes

Hjortebjerg et al. (2015) investigated the prognostic value of IGFBP-4 fragments in a cohort of Type 1 Diabetes (T1D) patients (3). A total of 178 T1D patients with diabetic nephropathy and 152 T1D patients with normoalbuminuria were prospectively followed up over a period of 12.6 years.

Following adjustments for nephropathy and traditional cardiovascular risk factors, high NT-IGFBP-4 and CT-IGFBP-4 levels remained prognostic of cardiovascular mortality with hazard ratios of 5.81 (P < .001) and 2.58 (P = .030) respectively (see Table 1). As a reference, the hazard ratio of the group of patients with NT- and CT-IGFBP-4 concentrations below threshold values was 1. In contrast, this study found no association with overall or cardiovascular death and adjusted levels of PAPP-A (see article for details).

For ROC (receiver-operating characteristics) analyses, all 330 T1D patients were used. The ROC area under curve [mean (95% confidence interval)] for NT-IGFBP-4 was 0.77 (95% confidence interval 0.71–0.84). For CT-IGFBP-4 it was 0.74 (95% confidence interval 0.66–0.81).

Table 1.
Relationship of NT-IGFBP-4 and CT-IGFBP-4 to cardiovascular mortality using Cox regression analyses.

		Cardiovascular Mortality	
		HR (95% CI)*	P Value
NT-IGFBP-4	Unadjusted	6.58 (3.75–11.57)	<.001
	Adjusted for nephropathy	4.13 (2.16–7.88)	<.001
	Adjusted for nephropathy and cardiovascular risk factors	5.81 (2.62–12.86)	<.001
CT-IGFBP-4	Unadjusted	4.90 (2.70–8.82)	<.001
	Adjusted for nephropathy	2.82 (1.43–5.55)	.003
	Adjusted for nephropathy and cardiovascular risk factors	2.58 (1.10–6.10)	.030

*HR: hazard ratio; CI: confidence interval

III. Hjortebjerg et al., 2017: Prediction of short-term to long-term mortality in patients with acute myocardial infarction

Hjortebjerg et al. (2017) investigated the levels of NT-IGFBP-4 and CT-IGFBP-4 as well as intact IGFBP-4 in 656 patients with ST-segment elevation myocardial infarction who were treated with percutaneous coronary intervention (7). Following multivariable adjustments, both NT-IGFBP-4 and CT-IGFBP-4 levels remained associated with all end points, including cardiovascular mortality with hazard ratios per doubling in protein concentration of 2.54 (95% confidence interval 1.59–4.07; P<0.001) and 2.07 (95% confidence interval 1.41–3.04; P<0.001) respectively. The incorporation of IGFBP-4 fragments into a clinical model with 15 risk factors improved C-statistics and model calibration, as well as providing incremental prognostic contribution, as assessed by net reclassification improvement and integrated discrimination improvement.

A Kaplan-Meier analysis showed that the majority of the deaths due to cardiovascular events occurred during the first three months (see Figure 5).

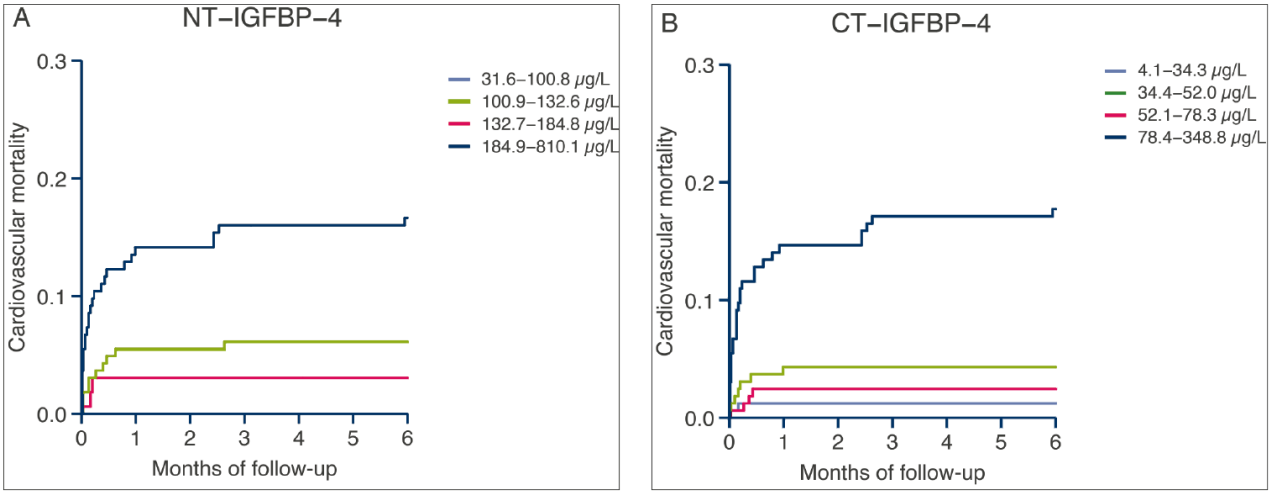

Figure 5.
A six-month cardiovascular mortality stratified by NT-IGFBP-4 (A) and CT-IGFBP-4 (B) cutoff values in patients with acute myocardial infarction; Kaplan–Meier analysis. *The figure was kindly provided by Dr. Rikke Hjortebjerg.*

IV. Postnikov et al., 2018: Prediction of short-term to medium-term all-cause mortality in patients with acute heart failure

As progressive heart failure is a major cause of morbidity and mortality following acute myocardial infarction, we suggested that IGFBP-4 fragments could also be utilized as biomarkers for the prognosis of heart failure (HF) outcomes (6). We evaluated the prognostic value of CT-IGFBP-4 for all-cause mortality in emergency patients with acute HF. CT-IGFBP-4 was measured at admission in the Li-heparin plasma of 156 emergency patients with acute HF. CT-IGFBP-4 predicted all-cause mortality at one month and one year follow-up periods: the areas under the ROC curves were 0.753 and 0.727 respectively. After adjustment for multiple clinical and echocardiographic variables CT-IGFBP-4 was an independent risk biomarker for one month and one year mortality (see Table 2).

Table 2.
Adjusted HR (95% CI) for all-cause mortality within one year and one month follow-ups by CT-IGFBP-4 levels in patients with acute heart failure.

	One month mortality		One year mortality	
	Hazard ratio, Univariate	Hazard ratio, Multivariate	Hazard ratio, Univariate	Hazard ratio, Multivariate
CT-IGFBP-4 ≥ 92.5 ng/ml	6.15 (2.12-17.79; p=0.0008)	5.39 (2.11-13.76; p=0.0004)	4.20 (2.11-8.39; p< 0.0001)	3.26 (1.63-6.51; p=0.0008)

REFERENCES

1. **Postnikov, A. B. 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, 519–524 (2012).
2. **Hjortebjerg, R. et al.** PAPP-A and IGFBP-4 fragment levels in patients with ST-elevation myocardial infarction treated with heparin and PCI. Clin. Biochem. 48, 322–328 (2015).
3. **Hjortebjerg, R. et al.** IGFBP-4 Fragments as Markers of Cardiovascular Mortality in Type 1 Diabetes Patients With and Without Nephropathy. The Journal of Clinical Endocrinology & Metabolism 100, 3032–3040 (2015).
4. **Konev, A. A. et al.** Characterization of endogenously circulating IGFBP-4 fragments—Novel biomarkers for cardiac risk assessment. Clinical Biochemistry 48, 774–780 (2015).
5. **Konev, A. A. et al.** Glycosylated and non-glycosylated NT-IGFBP-4 in circulation of acute coronary syndrome patients. Clin. Biochem. (2018). doi:10.1016/j.clinbiochem.2018.03.004
6. **Postnikov, A. B. et al.** C-terminal fragment of IGFBP-4 is independently associated with mortality in patients hospitalized due to acute heart failure. Poster presentation at ESC Heart Failure 2018, http://u.to/MmpyEg (P1828).
7. **Hjortebjerg, R. et al.** Insulin-Like Growth Factor Binding Protein 4 Fragments Provide Incremental Prognostic Information on Cardiovascular Events in Patients With ST-Segment Elevation Myocardial Infarction. J Am Heart Assoc 6, (2017).

PATENTS

Detection of IGFBP-4 Fragments as a Diagnostic Method

EP 2448969, US 9012610, US 9964549, JP 5840605, KR 101767611 and CN 102471379. This invention describes a method for diagnosing of cardiovascular diseases, which comprises detection of IGFBP-4 (insulin-like growth factor binding protein-4) fragments in patients' blood. It provides antibodies as well as epitopes for antibodies, specific to proteolytic fragments (both N- and C-terminal) of IGFBP-4 originated from IGFBP-4 molecule after its cleavage by specific protease PAPP-A. Antibodies could be used for development of immunoassay methods for quantitative or qualitative detection of IGFBP-4 fragments in human blood.

Method for Determining the Risk of cardiovascular Events Using IGFBP Fragments

US 10191066. This invention describes the method for determining the risk of future major adverse cardiovascular events, which comprises detection proteolytic fragments of IGFBP-4 or IGFBP-5 (insulin-like growth factor binding protein 4 or insulin-like growth factor binding protein 5) in patients' blood. The present invention provides antibodies and immunoas-says, suitable for specific measurement of proteolytic fragments of IGFBPs. In current invention the IGFBP fragments are suggested to be utilized as blood biomarkers for the risk prediction of major adverse cardiovascular events (MACE).

ORDERING INFORMATION

MONOCLONAL ANTIBODIES

Product name	Cat. #	MAb	Subclass	Remarks
Insulin-like growth factor binding protein 4	4IGF4	IBP3cc	IgG3	*In vitro*, EIA
		IBP163	IgG1	EIA
		IBP180	IgG2a	EIA
		IBP182	IgG2b	EIA

ANTIGENS

Product name	Cat. #	Purity	Source
Insulin-like growth factor binding protein 4, N-terminal fragment (NT-IGFBP-4), human, recombinant	8NGP4	8ILG4	Recombinant
Insulin-like growth factor binding protein 4, C-terminal fragment (CT-IGFBP-4), human, recombinant	8ILG4	≥90%	Recombinant

Please note that some or all data presented in this TechNotes has been prepared using MAbs produced *in vivo*. MAbs produced *in vitro* are expected to have similar performance.

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