New prognostic markers and the role of inflammation in the pathomechanism of chronic heart failure

Doctoral thesis

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1. Introduction

The opinion that chronic heart failure (CHF) mainly consists of haemodynamic alterations has largely changed. Nowadays, CHF is considered a complex syndrome; where the constant hypoperfusion, hypoxia and the alteration of the electrolyte homeostasis affect most of the organs, causing in long term a multi organ failure. Already in the early stadium of the disease chronic inflammation develops, the neurohormonal system is activated, vasoactive and natriuretic peptides are released, later the liver and renal function are impaired. Because of the peripheral edema and shear stress, endothelial dysfunction develops, the cell injury leads to stress response and the immune system becomes activated. The describing parameters of the above mentioned pathways are potential biomarkers, helping to understand the pathomechanism of the disease and to determine the therapeutic pathways and they have prognostic value.

1.1.Systemic inflammation

Several hypotheses have been suggested to describe the origin of immune activation in CHF. The myocardium itself can produce pro-inflammatory cytokines, and oxidative stress can also lead to immunactivation. Furthermore, according to the endotoxin hypothesis the edematous bowel wall has an increased permeability and the translocation of bacterial endotoxins from the gut into the circulation has been described in patients with CHF. Recent studies suggest that neurohormonal activation, such as of the renin-angiotensin-aldosteron system (RAAS), the adrenergic system and diminished cholinergic signaling could represent other mechanisms for immune activation and inflammation in CHF. The inflammation plays crucial role in the pathogenesis of the disease, while it leads to contractile dysfunction, increases the myocardial remodeling and contributes to the development of anemia and cardiac cachexia and increases the endothelial dysfunction. Thus the parameters describing the inflammation, like cytokine levels (tumor necrosis factor alpha (TNF-α), soluble TNF receptors (sTNF-R), interleukin 6 (IL-6)) show a strong correlation with

the severity of the disease, and they have predictive value in the outcome of the disease.

1.2. Vasoactive peptides

It is well known that in CHF the levels of vasoactive peptides increase. The most widely investigated are the B-type natriuretic peptide (BNP), and N-terminal-BNP (NT-proBNP) the inactive prohormone fragment produced equimolarly. Recently the two parameters are the most popular diagnostic and prognostic marker.

Endothelin-1 (ET-1) is one of the strongest vasoconstrictive peptide, its complex pleiotrop effect contributes to the progression of the disease. In patients with CHF the ET-1 levels are higher than in healthy controls, and it shows a strong correlation with symptoms and severity of the disease. Methodical developments provided reliable measurement of fragments of the ET-1 prohormon, C-terminal-proET-1 (CT-proET-1). CT-proET-1 is a promising prognostic marker, endothelin blockade may have a therapeutic potential in CHF. Since results are contradictory further validating studies are necessary.

The biological activity of adrenomedullin (ADM) in cardiovascular system is similar to the BNP, causing vasodilatation. Its effects similar to ET-1, modulates the immunsystem in several way. Plasma ADM levels are increased in patients with cardiovascular diseases, in example in CHF. Reliable measurement of ADM became available through the determination of the level of the inactive prohormone fragment, mid-regional-proADM (MR-proADM).

1.3. 70 kDa heat shock protein (Hsp70)

Hsp70 is traditionally considered as intracellular cytoprotective chaperone, and its level can increase several-fold in response to stress. It has been recognized, that Hsp70 are present in the peripheral circulation of healthy individuals (soluble Hsp70 – sHsp70). Elevation of serum Hsp70 was reported in patients with soft tissue trauma, with peripheral vascular disease, after acute myocardial infarction, preeclampsia and CHF. According to the literature data sHsp70 as an

ancient sign of stress can activate the immune system, and behave as a cytokine and chaperon simultaneously, called as ‘chaperokine’ function.

HspA1A and *HspA1B* are two of the human Hsp70 genes, they encodes the heat-inducible protein Hsp70. The genes are polymorphic, *HspA1B* A(1267) and *HspA1L* C(2437)T are well described, they are potentially accounting for variation in Hsp70 functions and susceptibility to stress tolerance. The association between the polymorphisms and sHsp70 level is not known, although in some diseases a more severe outcome was described in the presence of G and C alleles. Their roles in CHF were not studied yet.

1.4. Von Willebrand factor and its cleaving protease

Von Willebrand factor (VWF) plays a specific role in the homeostasis, it is stored in the Weibel-Palade bodies, it is a well known marker of endothelial dysfunction. The VWF is released from endothelial cells in an ultra large form (ULVWF), which distinguishes itself not only by molecular weight but also by the ability to aggregate platelets in conditions of high shear stress. The plasma metalloprotease ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif, 13) cleaves prothrombotic ULVWF into less active multimers. ADAMTS13 is produced by endothel and hepatic cells, in its absence the thrombotic events are more often.

Endothelial dysfunction is a well known complication of CHF, it causes elevation in VWF level, having a strong positive correlation with disease severity and has a prognostic value. The high VWF, and the disturbed inbalance between ADAMTS13 and VWF may play a role in the thromboembolic complications in CHF, although we have no information about the ADAMTS13 activity in the disease.

2. Aims

With a prospective cohort study of chronic heart failure patients we looked for the answers to the following questions:

1, Do MR-proADM and CT-proET-1 have a prognostic value in CHF for mortality and morbidity comparing with the „gold standard" NT-proBNP?

Several biomarkers help clinical decision making on patients with heart failure, N-terminal brain natriuretic peptide (NT-proBNP) being the most widely used and investigated one. However, multimarker strategies are emerging to provide complementary and additional information for this challenge. Among the new peptide markers novel vascular regulatory factors are increasingly investigated allowed by innovative and highly reliable assay methods. We aimed to investigate the correlation between the vasoactive MR-proADM, CT-proET-1 and disease severity and study the predictive value of the two peptides in mortality and morbidity.

2, What kind of biological correlations do the vasoactive peptides (NT-proBNP, MR-proADM and CT-proET-1) have in CHF?

Despite multiple recent studies focused on the biomarkers of CHF less is known about their biological correlates in the clinical setting. We aimed to investigate the *in vivo* biological correlations of the studied biomarkers in CHF patients.

3, Does any correlation exists between vasoactive peptides (MR-proADM és CT-proET-1) and inflammation, independently from disease severity?

In vitro and animal model studies provide some evidence that ADM and ET-1 play a role in the modulation of inflammation. Since the potential interaction between vasoactive peptides and inflammatory mechanisms has never been investigated in clinical setting, we aimed to study by statistical models, whether ADM and ET-1 levels are related to inflammatory markers in CHF independently from the progression of the disease.

4, Does any association exist between the stress reaction (described with sHSp70 level) and the severity of CHF? Does sHsp70 have any prognostic value?

According to literature data sHsp70 is elevated in CHF patients and correlates with the disease severity, but shows no predictive value. We aimed to validate the earlier results, and investigate the parameter of stress response in our patients and determine its role in the disease progression.

5, Is there any association between HspA1B A(1267)G, or HspA1L C(2437)T polymorphisms and the severity or outcome of CHF? Do the above mentioned polymorphisms show correlation with sHsp70 levels?

The Hsp70 gene is polymorphic, potentially accounting for variation in its functions and susceptibility to stress tolerance. The effect of the polymorphism on the outcome and severity of the disease it not known, whilst according to our best knowledge, the direct association with the serum Hsp70 level and the polymorphism also has never been reported. We aimed to investigate the above mentioned associations.

6, Does the sHsp70 level correlate with inflammatory markers? What kind of biological correlation does sHsp70 level have in CF patients?

Serum Hsp70, as a marker of cellular stress may play a role in the systemic inflammation observed in CHF, although we do not have date about the association between cytokines and sHsp70 level. The aim of our study was to identify the biological correlates of Hsp70 serum levels in patients with CHF.

7, Do the VWF and the ADAMTS13 activity differ in CHF patients compared to healthy individuals?

Thromboembolic complications in CHF patients may be caused by the endothelial dysfunction and the increase of VWF levels. According to our best knowledge ADAMTS13 activity has never been studied in CHF, although the change in ADAMTS13/VWF ratio may have clinical relevance. We aimed to determine for the first the ADAMTS13 activity in our CHF patients.

8, What kind of biological correlations do VWF and ADAMTS13 activity have in CHF patients? How do the two protein level and activity change during the disease progression?

To understand the changes and regulation of VWF.Ag level and ADAMTS13 activity it is important to investigate their associations with clinical and laboratory parameters.

9, Does ADAMTS13/VWF ratio have any prognostic value in mortality, rehospitalization or in thromboembolic complications?

It is reported that high VWF level is associated with a worse outcome, but our knowlage about the association between VWF and thromboembolic complication is limited. Because of the biological function of ADAMTS13, we hypothised that ADAMTS13 activity would increase the predictive value of VWF.

3. Methods

The study was carried out at the IIIrd Department of Internal Medicine, Semmelweis University. A total of 195 patients (147 men, 48 women), all white Caucasians, were enrolled consecutively with the signs of CHF, and with a left ventricular ejection fraction less than 45%, determined by trans-thoracic echocardiography, independently of the etiology of the disease, both from the out- or inpatient cardiology departments. The full clinical record of the patients was registered at inclusion with the detailed physical status and results of routine laboratory tests. Blood samples were taken, serum-, plasma and DNA bank were built. All patients were contacted after 1-year from study entrance, all major clinical events (re-hospitalization due to worsening of CHF, mortality, the presence of thromboembolic complications, or lack of them) were registered.

Levels of TNF TNF-α, IL-6, sTNF-R, sHsp70, VWF:Ag and NT-proBNP were determined by ELISA kits, according to the manufacturers instruction. Fragments of the ADM and ET-1 prohormones with the greatest stability (MR-proADM, CT-proET-1) were measured using sandwich immunluminometric assays (BRAHMS AG., Hennigsdorf, Germany). A fluorigenic substrate FRETS-VWF73 was applied for determination of ADAMTS13 enzyme activity. The HspA1B and HspA1L single nucleotide polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism technique.

Statistical analyses were carried out using the software STATISTICA (StatSoft Inc., Tulsa, USA), SPSS (SPSS Inc., Chicago,USA), MedCalc (MedCalc Software, Mariakerke, Belgium), and Prism for Windows (GraphPad Software, San Diego, USA). Non-parametric test were used, two-tailed p values were calculated and the significance level was put at a value of $p<0.05$.

4. Results

The participants belonging to NYHA functional classes III and IV had significantly higher levels of inflammatory parameters, worse renal function, impaired synthetic liver capacity, higher levels of vasoactive proteins (NT-proBNP, MR-proADM, CT-proET-1), higher sHsp70 and VWF levels, while the ADAMTS13 activity was significantly lower, as compared to patients in NYHA I and II classes. During the first year of follow up 36 patients died and 41 patients were rehospitalized because of the progression of the disease (together 'event' of 'combined end point'). The difference between patients with event free survival, and patients with events, were similar like between groups divided according to the severity of the disease.

MR-proADM and CT-proE-1 levels showed strong positive correlation with the disease severity markers (Kruskal-Wallis ANOVA $p<0.00001$). Patients with high vasoactive peptide levels If vasoactive peptide levels were stratified according to tertiles, patients with MR-proADM and CT-proET-1 levels in the highest tertile had a 8.09 (95% CI 3.59-18.20) and 5.71 (2.61-12.51) fold risk, respectively, to have advanced heart failure (NYHA III-IV), when compared to patients belonging to the lowest tertile group. According to our results MR-proADM and CT-proET-1 levels showed a strong positive correlation with inflammatory parameters (CRP, TNF-α, sTNF-R, IL-6), and the association remained significant even in logistic regression models after an adjustment for parameters describing the disease severity. Analyzing the receiving operating characteristic (ROC) graphs of MR-proADM, CT-proET-1 and the 'gold standard' NT-proBNP for predicting mortality or event free survival, we found a similar area under the curve (AUC). In multivariable Cox regression models, adjusting for key clinical covariates, high MR-proADM and high CT-proET-1 were independent, significant predictors of mortality and event free survival ($HR_{MR\text{-}proADM}$ 1.50 [CI 1.23-1.82], $HR_{CT\text{-}proET\text{-}1}$ 1.65 [CI 1.23-2.34]). After adjustment for NT-proBNP, the models remained significant ($HR_{MR\text{-}proADM}$ 1.32 [CI 1.05-1.65], $HR_{CT\text{-}proET\text{-}1}$ 1.46 [CI 1.01-2.11]).

Serum Hsp70 levels showed a strong positive correlation with disease severity markers (Spearman rank correlation, Kruskal-Wallis ANOVA $p<0.005$). There was no association between *HspA1B* and *HspA1L* alleles and sHsp70 levels in the patients. An allelespecific interaction was found between sHsp70 levels and NYHA classes.The association of variant allele G of HspA1B with increased sHsp70 levels was present mainly in patients with the most severe disease (factorial ANOVA $F=5.45$, $p<.008$). In Cox regression analysis sHsp70 levels did not have any predictive value for mortality of event free survival. In our coholrt sHsp70 showed a strong positive correlation with the markers of cell injury (γGT, LDH, $p<0.01$, $r>0.25$), while in an unexpected way, it showed no association with inflammatory cytokines (TNF-α, IL-6, CRP, $r<0.16$)

The mean ADAMTS13 activity was decreased (76.7%, SD 27.6%, while it is 100% in healthy blood donors). ADAMTS13 activity showed a strong negative association with disease severity, while VWF correlated in the opposite way. It suggests an imbalance between the two proteins, which may have clinical relevance. In multivariable Cox regression models the low ADAMTS13/VWF ratio was a significant independent predictor of clinical events (HR 1.94 [CI 1.3-2.9]). Patients suffering from thromboembolic or vascular complications during the first year of follow up had significant lower baseline ADAMTS13 activity($p=0.012$). ADAMTS13 showed the strongest correlation with parameters describing the synthetic capacity of the liver (albumin, total protein, $r>0.25$, $p<0.003$).

5. Conclusions

1, High MR-proADm and high CT-proET-1 have a similar predictive value for the widely used NT-proBNP in mortality and for combined end point including mortality and rehospitalization. CT-proET-1 may be more useful in predicting mortality, while MR-proADM may be more advantageous in predicting event free survival like NT-proBNP. Both new vasoactive peptides may be new, useful prognostic marker.

2, Since MR-proADM and CT-proET-1 show strong correlation with parameters describing the severity of heart failure, can be useful biomarkers. Like NT-proBNP, after further research they can help in the diagnosis, follow up of CHF patients and can guide therapy. Their correlation with renal function, age and body mass index may be warning about their general use.

3, MR-proADM and CT-proET-1 levels correlate with the markers of systemic inflammation independently from disease severity. The mutual regulation between vasoactive peptides and inflammatory mechanism may play role in the progression of the disease.

4, Serum Hsp70 level is increasing in patients with severe heart failure, and it correlates with disease severity. It has no predictive value for mortality and morbidity in CHF.

5, The genetic polymorphisms of Hsp70 gene (*HspA1B A(1267)G* and *HspA1L C(2437)T*) show no correlation with the severity and outcome of HF. No primer association exists between sHsp70 levels and the allele frequency of the two polymorphisms, but in advanced CHF G allele carriers have higher sHsp70 concentration.

6, In CHF sHsp70 shows no correlation with inflammatory markers, but it associated significantly with parameters of cell- and tissue injury. Serum Hsp70 may be the marker of cell integrity.

7, Levels of VWF increase in CHF, while the VWF cleaving protease, ADAMTS13 activity decreases compared to healthy controls. This way the balance between the two proteins is disturbed, the ADAMTS13 and VWF ratio

significantly decrease with the progression of the disease. Both variables and their ratio correlate significantly with the parameters describing disease severity.

8, In CHF patients ADAMTS13 activity showed the strongest correlation with parameters describing the synthetic capacity of the liver, while it does not correlated with acute phase proteins. The low ADAMTS13 activity may be due to the impaired hepatic capacity, since the liver is one of the sources of ADAMTS13.

9, In CHF the low ADAMTS13/VWF ratio is an independent significant predictor of mortality and rehospitalization. Patients suffering from thromboembolic complications have significant lower baseline ADAMTS13 activity. According to our results the change in ADAMTS13 activity and VWF level may play a role in thromboembolic complications in CHF patients.

6. Publication list

6.1. Publications connected to the thesis

1, Gombos T, Förhécz Z, Pozsonyi Z, Jánoskuti L, Prohászka Z. Interaction of serum 70-kDa heat shock protein levels and HspA1B (+1267) gene polymorphism with disease severity in patients with chronic heart failure.
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Thromb Haemost 2009 Sep;102(3):573-80. IF: 3,803 (2008)

6.2. Other publications

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