# RESEARCH OF PROGNOSTIC MARKER IN CARDIOVASCULAR DISEASES

**PhD thesis**


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# 1. Introduction

## 1.1. Post cardiac arrest state

Cardiac arrest causes generalized ischemia/hypoxia, and subsequent resuscitation inflicts reperfusion injury. This process initiates a complex series of events, known as the post-cardiac arrest syndrome. Hypoxia and the reperfusion injury cause acute stress response in the brain. Although all the organs are damaged by hypoxia, cerebral injury occurs first, because the ischemia tolerance and metabolic reserve of the brain are limited. Ischemia and reperfusion injury cause intense stress in the brain by multiple pathways, including oxidative stress, microvascular injury, excitotoxicity, blood–brain barrier dysfunction, postischemic inflammation initiated by neuronal, glial and endothelial cell death, or apoptosis.

## 1.2. Heart failure

Heart failure defined, clinically, as a syndrome in which patients have typical symptoms (e.g. breathlessness, ankle swelling, and fatigue) and signs (e.g. elevated jugular venous pressure, pulmonary crackles, and displaced apex beat) simultaneously, resulting from an abnormality of cardiac structure or function. Nowadays heart failure (HF) is a major public health problem associated with increased morbidity and mortality. Heart failure has traditionally been considered a disease of the myocardium, with symptoms arising from altered hemodynamic. However, it is now recognized that, in addition to the marked neuroendocrine disturbance, there is a perturbation of cytokine expression in patients with heart failure. The resulting inflammatory imbalance plays a central role in the underlying pathophysiological processes of heart failure.

### 1.3. HSP70

Within the heat shock protein (Hsp) family, HSP70 (HSPA1A) is a structurally and functionally conserved protein in evolution. HSP70 plays multiple roles in cellular homeostasis. Its level increases rapidly in response to various types of severe stress, as a protection against a subsequent, nearlethal, ischemic or hypoxic event. Previously, human heat shock proteins were regarded as obligate intracellular molecules that essentially contribute to survival by acting as molecular chaperones. Subsequently, due to the development of protein analytic method, HSP70 has been detected also in the circulation, in the extracellular space, and its presence has been demonstrated in the serum of healthy individuals. HSP70 could be passively released from necrotic cells, and actively excreted by a non-classical secretory pathway.

Additionally, extracellular HSP70 was considered among the intercellular signalling regulators of inflammation. Elevated HSP70 in the serum or tissues appears to be a nonspecific indicator of organ ischemia or dysfunction.

### 1.4. The complement system

Complement is an important component of the humoral immune system. It represents a highly effective means for the destruction of invading micro-organisms, for the elimination of immune complexes, as well as for the clearance of damaged host cells. Complement is activated by three pathways: the antibody-dependent classical pathway, the alternative pathway or the recently discovered ficolin-/mannose-binding lectin pathway. Complement activation leads to the formation of opsonins (C3b), anaphylatoxins (C3a and C5a) and the cytolytic membrane attack complex

(C5b9). A large body of evidence from the literature shows that complement activation plays an important role in the pathophysiology of cell and tissue damage after ischaemia and reperfusion. Accordingly several ischaemic stroke and post cardiac arrest studies reported complement activation.

### 1.5. RDW

The red blood cell distribution width (RDW) is a measure of the variation and an index of the heterogeneity of red blood cell (RBC) volume that is reported as part of a standard complete blood count and used in the differential diagnosis of anaemia. RDW is calculated by dividing the standard deviation (SD) of RBC volume by the mean corpuscular volume (MCV) and multiplying by 100 to express the result as a percentage, i.e., RDW = [Standard deviation of MCV/mean MCV] × 100.

Red blood cell distribution width has emerged as a new prognostic biomarker in cardiovascular diseases. Highly significant associations have been described between RDW value and mortality in patients with coronary artery disease, acute and chronic heart failure as well as in the general population.

### 1.6. Statistical analysis of prognostic factors

Identification of risk factors is one of the most frequent questions in medical research currently. Several reports showed “significant” and “independent” prognostic factors in a variety of human conditions, however, those were not tested about predictive information in addition to standard risk markers. Out of the promising candidate prognostic factors only few have really usefulness in the clinical practice. In the past years different statistical method has been developed to identify risk factors and

test its influence. Nowadays in the medical practice the c-statistics and COX regression are the most important analysis.

We know however, that they can be considerably insensitive in several cases. Hence, recently novel statistical approaches (reclassification) have been developed to test the performance and usefulness of new risk factors and prognostic markers. With help of the NRI (net reclassification improvement) and the IDI (integrated discrimination improvement) we can better assess the discrimination capacity of the new prognostic markers in the global prognostic model. NRI and IDI make possible to test properly the influence of the new prognostic marker in the new prognostic model (with the new prognostic marker) compared to the previous prognostic model (without the new prognostic marker).

## 2. Objective

We intended to answer the following questions by studying two well-characterized populations of patients with cardiovascular diseases (post cardiac arrest and chronic heart failure).

*2.1. Is there any relationship between the levels of extracellular HSP70 and the short term outcome of post cardiac arrest patients?*

We hypothesized that HSP70 might prove not only a reliable biomarker of neuronal damage, but also a good surrogate of endothelial-cell activation and death, as well as of inflammatory reaction in post-cardiac-arrest patients. We further assumed that as an integrative marker of stress, HSP70 could be an independent predictor of mortality in these critically ill subjects. Accordingly, the aim of the present study was to describe the HSP70 response in post-cardiac-arrest patients undergoing mild hypothermia treatment and to analyse its association with the overall survival.

*2.2. Is there any relationship between the levels of complement activation and the short term outcome of post cardiac arrest patients?*

Our group demonstrated previously that complement activation is associated with unfavourable outcome after acute ischaemic stroke and chronic heart failure (CHF). We further presumed that as an integrative marker of cellular damage, complement activation might be an independent predictor of mortality in post-cardiac arrest patients. Accordingly, our study aimed to describe the changes of complement proteins and activation markers in post-cardiac arrest patients undergoing mild therapeutic hypothermia and to analyse its association with overall survival.

*2.3. Is there any relationship between the levels of RDW and the long term outcome of patients with heart failure?*

As an integrative marker of inflammation, ineffective erythropoiesis, undernutrition and impaired renal function and based on its relationship to 1-year mortality, we hypothesized that RDW could be an independent predictor of long-term mortality in our HF cohort. The present study aimed to analyse the association of RDW values with overall 5-year survival in CHF.

*2.4. Is there any relationship between the levels of extracellular HSP70 and the long term outcome of patients with heart failure?*

As an integrative marker of stress, and based on its relationship to disease severity, we hypothesized that HSP70 could be an independent predictor of mortality in HF. Accordingly, the present study aimed to analyze the association of HSP70 levels with overall 5-year survival.

*2.5. Which promising new prognostic factor has really, statistically justifiable, clinical usefulness in heart failure?*

Using reclassification based novel statistical approaches the present study aimed to describe the features and compare the promising, previously significant, new biomarkers with each other.

# 3. Methods

*3.1. Study subjects*

***Post cardiac arrest study***

We studied blood samples obtained between 2009 and 2011 from 46 (38 males, 8 females, median age: 64 years) consecutive comatose patients resuscitated succesfully after cardiac arrest at the Semmelweis University Heart and Vascular Center. All patients underwent diagnostic coronary angiography and percutaneous coronary intervention (PCI), where indicated. After admission to the intensive care unit all patients were cooled to 32–34 °C in the frame of the protocolised intensive care. We used a water-circulating blanket system (Blanketroll III, Cincinnati Subzero Medical Division, Cincinnati, OH, USA) to achieve controlled hypothermia. All patients were followed-up for 30 days.

The age- and sex-matched control group comprised 46 stable cardiovascular patients (38 males, 8 females, median age: 63 years) treated with atrial fibrillation.

***Heart failure study***

We studied blood samples obtained between 2005 and 2007 from 195 (145 males, 50 females, median age: 69 years) heart failure patients with <45% left ventricular ejection fraction (EF) at the Semmelweis University IIIrd Department of Internal Medicine. Consecutive patients with clinical suspicion of CHF referred to transthoracic echocardiography were considered for inclusion. The full clinical record of the patients was registered at inclusion with the detailed physical status and routine laboratory tests. Blood samples were taken after 12 h of fasting. All patients were followed-up for 60 months.

In both study (Post cardiac arrest study and Heart failure study) during follow-up, the clinical and laboratory findings accumulated from the subjects have been recorded in a database, as well as a serum, plasma bank has been established using the blood samples. The samples were stored at −80 °C until analysis.

*3.2. Laboratory methods*

Serum and plasma samples were stored at minus 80 °C until processing. Commercial ELISA techniques were used to determine the concentrations of HSP70, TNFα, sICAM-1, S100B, big endothelin-1, CRP, C3 and C4 in serum. The levels of vWF, C3a, C4d, SC5b9 and Bb were determined in EDTA plasma, using in-house or commercial ELISA techniques.

*3.3. Statistical analysis*

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, US), SPSS version 13.0 (SPSS Inc., Chicago, IL, US) open source R software (R version 2.15.0, 2012-03-30) and STATISTICA version 8.0 (StatSoft Inc. Tulsa OK, US). All statistical tests were two-tailed, and the results were considered statistically significant when p-value was $<0.05$.

## 4. Results

*4.1. The relationship between the levels of extracellular HSP70 and the short term outcome of post cardiac arrest patients*

On admission, HSP70 levels were higher in survivors and non-survivors (1.31 [0.76 – 2.73] and 1.70 [1.20- 2.37] ng/mL) than in controls (0.59 [0.44 - 0.83] ng/mL), $p < 0.001$), but there was no difference between HSP70 levels in survivors and in non-survivors. We noticed a statistically significant trend of decrease in HSP70 concentrations in the survivors at 6 hours 0.70 (0.38 - 1.78) ng/mL, and at 24 hours 0.56 (0.16 - 0.71) ng/mL in comparison to the baseline values. However, this decline was not observed in the non-survivors either at 6 hours 1.70 (0.65 - 2.70) ng/mL, or at 24 hours 1.54 (0.79 - 1.72) ng/mL. At 24 hours, HSP70 levels were similar in the survivors and in the control group, whereas a significant elevation was observed in non-survivors (as compared to controls).

Kaplan-Meier survival analysis was done to describe mortality over time in the subsets of patients stratified according to the median HSP70 level (0.74 ng/mL). In our study, higher HSP70 levels were associated with an increased mortality (Log-rank test, $p < 0.001$).

In the multivariable Cox analysis, the prediction of 30-day mortality by HSP70 was adjusted for age, sex and severity (APACHE II score). HSP70 remained an independent, significant predictor of mortality (hazard ratio =6.996, 95% confidence interval 2.254 – 21.717, for comparison of patients with high or low HSP70 level).

*4.2. The relationship between the levels of complement activation and the short term outcome of post cardiac arrest patients*

We observed a significant change in the levels of complement activation products. Bb, sC5b9, and C3a/C3 ratio (a measure of C3 activation) decreased significantly during the initial 24 hours of therapeutic hypothermia. We found a consistently significant difference only in C3a/C3 ratio between the groups (survivors and non-survivors); which suggests an association with the outcome. At each time point, the C3a/C3 ratio was higher in non-survivors than in survivors. The most important difference was observed at 24 hours after the initiation of therapeutic hypothermia.

The 46 patients were stratified according to the median of C3a/C3 ratio (measured at 24 hours), into low- or high- C3a/C3 ratio groups. Kaplan-Meier survival analysis was done to evaluate mortality over time in both groups. In our cohort, higher C3a/C3 ratio was associated with increased mortality (Log-rank test, $p = 0.0043$). C3a/C3 ratio, age, and the severity score were also associated with 30-day mortality. Therefore, we performed multivariate Cox proportional hazards regression analysis to test the potential influence of confounders on the predictive role of the C3a/C3 ratio. In the multivariable Cox model, the prediction of 30-day mortality by the C3a/C3 ratio was adjusted for age, sex, and APACHE II severity score. The C3a/C3 ratio was an independent, significant predictor of mortality in adjusted models (hazard ratio = 2.960, 95% confidence interval 1.095 – 7.998, for comparison of patients with high or low C3a/C3 ratio).

*4.3. The relationship between the levels of RDW and the long term outcome of patients with heart failure*

We found that RDW was 14.0% (13.4-14.7%) in survivors, and 14.9% (14.1-16.4%) among non-survivors, and the difference was significant ($p < 0.001$). The 195 patients were stratified, according to the median value of RDW (14.5%), into low- or high-RDW groups. Kaplan-Meier survival curves were plotted to evaluate mortality over time in both groups. In our cohort, higher RDW values were significantly associated with decreased 5-year survival (Log-rank test, $p <0.001$).

In on our previous analyses age, impaired renal function, low BMI (body mass index), elevated diastolic blood pressure, decrease in sodium or haemoglobin, and elevation in NT-proBNP levels associated with poor 1-year mortality. Therefore, we performed multivariate Cox proportional hazards regression analysis to test the potential influence of confounders on the predictive role of the RDW. In multivariate Cox model, the prediction of 5-year mortality by RDW was adjusted for age, renal function (GFR), haemoglobin, BMI, diastolic blood pressure, sodium and NT-proBNP. RDW remained an independent, significant predictor of 5-year mortality in adjusted model (hazard ratio = 1.639, 95% confidence interval 1.074 – 2.499, for comparison of patients with high or low RDW level).

*4.4. The relationship between the levels of extracellular HSP70 and the long term outcome of patients with heart failure*

We analysed whether increased HSP70 levels are associated with all-cause mortality, and found a significant ($p = 0.0101$) association. The 195 patients were stratified, according to the median value of HSP70 (0.33 ng/ml), into low- or high-HSP70 groups. Kaplan-Meier survival analysis was done to describe mortality over time in both groups. In our study, higher HSP70 levels were associated with an increased mortality (Log-rank test, $p = 0.0047$).

In addition to HSP70 levels, we found that age, BMI and left ventricular EF, as well as NT-proBNP, and serum creatinine levels were also associated with 5-year mortality. Therefore, multivariable Cox regression analysis was performed to test the independence of HSP70 from other covariates. In the multivariable Cox analysis adjusted for age, sex, BMI, serum creatinine, and NT-proBNP, HSP70 remained a significant predictor of 5-year mortality (hazard ratio = 1.556, 95% confidence interval 1.042-2.325 for comparison of patients with high or low HSP70 level).

*4.5. Clinical usefulness of promising new prognostic factor in heart failure*

In the multivariate Cox proportional hazards regression analysis both RDW and HSP70 were significant predictors of 5-year mortality. The AUC of the ROC curves (c-statistic) increased using RDW and HSP70 in the new models, however, there were no statistically significant differences (from 0.752 to 0.772; from 0.754 to 0.769, respectively). Calculating NRI we found significant improvement as new prognostic factors were incorporated in the reference models ($NRI_{RDW}$ = 0.383 and $NRI_{HSP70}$ = 0.314). Although the values of IDI were also positive at both cases we found significant improvement only in the case of RDW ($p = 0.027$). Testing calibration we observed that the model with RDW was well calibrated, however, the model with HSP70 was not, the calibration slope graphically was far away from the 45-degree line.

Using calibration and reclassifications tests we established that incorporating red cell distribution width to the reference model may be clinically useful in the assessment of long term prognosis of patients with heart failure, however the role of the HSP70 is questionable, and before the final judgement further independent studies needed.

# 5. Conclusions

## 1. Research of early prognostic markers in post cardiac arrest patients

*The prognostic role of HSP70*

According to our study the persistently high level of extracellular HSP70 during the initial 24 hours of therapeutic hypothermia is an independent prognostic marker of 30-day mortality in post-cardiac-arrest patients.

*The prognostic role of complement activation*

In accordance with previous publication we observed complement activation in post-cardiac arrest patients, and the activation marker C3a/C3 ratio is associated with 30-day survival. The C3a/C3 ratio determined at 24 hours predicted 30-day mortality regardless of age, sex, and APACHE II score.

## 2. Research of long-term prognostic markers in patients with heart failure

*The prognostic role of RDW*

Based on our study we concluded, that the RDW is a new, long-term prognostic marker in patients with heart failure which is independent of age, renal function, haemoglobin, diastolic blood pressure, serum sodium and NT-proBNP level. The elevated level of RDW is associated with worse outcome in patients with heart failure. Using RDW in the prognostic model of this disease the model improved significantly, this improvement was tested exactly by reclassification methods.

*The prognostic role of HSP70*

We found that the HSP70 is a significant, long-term prognostic marker in patients with heart failure which is independent of age, sex, BMI, creatinine level and disease severity (NT-proBNP or left ventricular EF). The elevated level of HSP70 is associated with worse outcome in patients with heart failure in the 5-year follow-up. Using HSP70 in the prognostic model of this disease the model did not improve significantly as tested by reclassification methods.

**3. Clinical usefulness of new prognostic markers**

The small sample size in the post cardiac arrest study did not make possible to test the improvement of the prognostic model.

In the heart failure study we observed improvement in the prognostic model, using RDW or HSP70 as new prognostic marker. In both models the Nagelkerke's $R^2$ and Brier scores showed better model performance incorporating the new prognostic markers (RDW or HSP70) in the reference models. Calculating IDI and NRI we found that only RDW is a promising biomarker in heart failure, which improves the prognostic model. Adjusted Cox regression showed that HSP70 associated significantly with the outcome, however, this not means directly better prognosis assess, clinical usefulness. Incorporating HSP70 in the prognostic model we found worse calibration, and no improvement in the discrimination (IDI was not significant) although model performance and the NRI were significant.

Interpretation of results on new prognostic factors has to be done carefully, and only appropriate reclassification approaches may help to confirm the clinical usefulness.

# 6. List of publications

*6.1. Publications related to the thesis*

1) Jenei ZM , Zima E , Csuka D , Munthe-Fog L , Hein E , Szeplaki G , Becker D , Karadi I , Prohaszka Z , Garred P , Merkely B. (2014) Complement activation and its prognostic role in post-cardiac arrest patients. Scandinavian Journal of Immunology, 79:(6) 404-409.
(2014) IF: 1.882*

2) Jenei ZM , Szeplaki G , Merkely B , Karadi I , Zima E , Prohaszka Z. (2013) Persistently elevated extracellular HSP70 (HSPA1A) level as an independent prognostic marker in post-cardiac-arrest patients. Cell Stress & Chaperones, 18:(4) 447-454.
(2013) IF: 2.537

3) Jenei ZM , Gombos T , Foerhecz Z , Pozsonyi Z , Karadi I , Janoskuti L , Prohaszka Z. (2013) Elevated extracellular HSP70 (HSPA1A) level as an independent prognostic marker of mortality in patients with heart failure**.** Cell Stress & Chaperones, 18:(6) 809-813.
(2013) IF: 2.537

4) Jenei ZM, Prohaszka Z. (2013) Hasznos tesztek az új prognosztikai markerek klinikai értékének felmérésére: a szívelégtelenség példája: [Useful tests to assess the clinical usefulness of new prognostic markers: the example of heart failure]. Orvosi Hetilap, 154:(35) 1374-1380.

6.2. Other publications

1) Kovacs E , Jenei ZM , Horvath A , Geller L , Szilagyi S , Kiraly A , Molnar L , Sotonyi Jr P , Merkely B , Zima E. (2011) A hypothermia élettani hatásai [Physiologic effects of hypothermia]. Orvosi Hetilap, 152:(5) 171-181.

** The impact factor of the marked journal at the labeled years is not available*

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