Investigating the role of haematological and inflammatory factors in the pathogenesis of chronic heart failure

PhD Thesis

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1. INTRODUCTION

Currently, cardiovascular diseases are responsible for more than half of all deaths. The most common manifestations of these fatal heart diseases are ischaemic heart disease and heart failure

According to a survey based on the Hungarian National Health Insurance database, the average prevalence of heart failure in Hungary between 2004 and 2010 was 1.6%, with a prevalence of around 15-20% over the age of 80.

Unfortunately, even with state-of-the-art diagnostic methods and evidence-based, well-chosen therapy, the survival of heart failure patients in the fifth year after diagnosis is no more than 50%.

All these data make it vital to have a broad understanding of and research into the diagnostic and therapeutic options for this disease.

In order to manage chronic heart failure more effectively, it is essential to determine the severity and prognosis of the disease, on the basis of which, patients with more severe conditions requiring special treatment can be selected. In order to classify the risk as accurately as possible, research into new markers that determine prognosis and the underlying pathophysiological processes is of paramount importance, as it offers the possibility of identifying new therapeutic targets.

2. OBJECTIVES

The aim of the study is to investigate biomarkers that determine the prognosis of chronic heart failure. Particular emphasis was placed on characterizing the additional features of RDW and anemia, as well as the prevalence, role in pathogenesis and association of inflammatory markers. Given the existence of multiple treatment strategies for the medical management of patients with chronic heart failure, there is now a particular need to identify biomarkers associated with key processes of pathogenesis and to characterise their features.

In response to this challenge, my doctoral work involved the implementation of a prospective cohort study specifically designed to investigate the pathogenesis and markers of the disease and to identify factors associated with its outcome.

The specific objectives of my thesis were formulated around the following questions:

1. What is the relationship between heart failure severity and markers of anaemia and inflammation?

My aim was to define and validate the prognostic role of RDW in a prospective cohort study.

2. What is the prognostic value of RDW in terms of mortality in patients with chronic heart failure? How strong is the association between RDW and mortality, and what is the relative weight of RDW compared to the known prognostic factors?

Knowing the markers that determine the severity of heart failure can be used to select patients with more severe conditions requiring special treatment, my aim was to explore the predictive value of RDW alongside known prognostic factors for survival and rehospitalisation.

3. What are the biological links and correlates of RDW in chronic heart failure?

Prior to my study, the pathophysiological processes underlying RDW changes in chronic heart failure had not been investigated.
Therefore, my aim was to understand the pathophysiological processes leading to changes in RDW by exploring the relationship between RDW and the main marker and mediator levels that characterise the disease.

Endothelial dysfunction plays an important role in the background of chronic heart failure progression, and therefore I also investigated the following question:

4. What is the prognostic role of the von Willebrand factor level on mortality in chronic heart failure at short- and long-term follow-up? How close is the association between the von Willebrand factor and mortality in chronic heart failure, given the known prognostic factors The activation of inflammatory processes is of great importance in the pathogenesis and progression of chronic heart failure, and little is known about the role of the complement system, an important part of the natural immune system. At C3 complement, the classical, alternative and lectin activation pathways converge, which is why I have focused on the following question:

5. What is the prognostic role of anaphylatoxin C3a in the prognosis of chronic heart failure? What are the biological relationships and correlates of anaphylatoxin C3a in chronic heart failure?

Given the design of the study, I used two types of statistical procedures to achieve the objectives. Multivariate logistic regression models were used to characterise the relationships between pathogenetic factors, while Cox proportional hazards models were used to examine the temporality of the study endpoints (rehospitalisation, mortality). This strategy allowed differences in baseline characteristics between study groups to be smoothed out at the analysis stage.

3. METHODS

3.1 Patient population

Between February 2005 and July 2006, 195 consecutive patients were enrolled in our prospective, follow-up study at the Department of Internal Medicine, Semmelweis University.

The study protocol complied with the Declaration of Helsinki and was approved by the Semmelweis University Regional, Institutional Scientific and Research Ethics Committee (TUKEB 230-42/2006-1018EKU).

After appropriate verbal and written information, patients were included after signing a consent form.

Inclusion criteria included symptoms of heart failure and left ventricular ejection fraction below 45% confirmed by transthoracic echocardiography. Exclusion criteria were acute infection and known malignancy (13 patients were excluded from the study for this reason).

A physical examination was performed at the same time as the full clinical record was completed (detailed anamnesis, key patient details and contact information for follow-up).

Blood sampling was taken in the morning, after 12 hours of fasting, by puncture of the antecubital vein using a closed blood collection system. After appropriate preparation, serum and plasma aliquots were prepared from a native blood sample, coagulated with EDTA and sodium citrate, and stored at -70 ˚C until further analysis.

The first visit took place between 12 and 18 months after admission (on average 14.5 months). The primary endpoint was mortality and hospital admission (rehospitalisation) due to worsening heart failure. If both events occurred in the patient, rehospitalisation was documented as the primary endpoint (n=8). For all patients, we considered the follow-up (censoring) period to be 365 days from the start of the study.

The visit was conducted in person or by telephone interview. In the absence of the former, events were recorded using the patient record system.

3.2. Laboratory measurements

Clinical chemistry and biochemical laboratory parameters were measured by Roche Integra 800 analyzer, and hematologic variables were determined by Cell.Dyn 3500 hematology analyzer. Levels of serum EPO (Roche Diagnostica, Mannheim, Germany), tumor necrosis factor alfa (TNF-α), interleukin-6 (IL–6), soluble TNF receptor I (TNF-RI), TNF receptor II (TNF-RII) (R and D Systems, Minneapolis, Minnesota, USA), az N-terminal pro-brain natiurwetic peptide (NT-proBNP) (Biomedica Gruppe, Wien, Austria), von Willebrand factor antigen (vWF-Ag) (DakoCytomation,Glostrup, Denmark) were determined by a technique of ELISA (Enzyme-Linked Immun SorbentAssay), based method using a protocol following the manufacturers' instructions.

Serum C3 and C4 levels were measured using a RocheIntegra 800 automated immunoturbidimetric method (Tina-quant®), while C3a and sC5b-9 complement activation product determinations were performed from EDTA plasma using ELISA Kits (Quidel, San Diego, California, USA) using the protocol according to the manufacturers' instructions.

3.3 Statistical analysis

Because most of the variables showed skewed distributions, desciptive statistics and non-parametric tests are presented as medians with interquartile ranges or percentages.

For comparisons of continuous variables between two independent samples, the non-parametric Mann-Whitney U test was used, and for three or more groups, non-parametric analysis of variance (Kruskal-Wallis) was performed. Pearson $\chi 2$ test was used to explore the relationship between categorical variables. Spearman's rank correlation coefficient was used to examine the correlations between variables, while multiple linear regression was calculated using the logarithmic values of the variables.

Kaplan-Meier curves were constructed to plot events during follow-up, and differences between groups were analysed using the Logrank test. Survival was calculated by determining the time from inclusion to endpoint (death or hospital admission for heart failure).

Survival analysis was performed using univariate and multivariate Cox regression analysis. To build the multivariate model, the chi-squared values determined by univariate Cox regression analysis were used to select the best predictors for the subsequent multivariate regression model. To facilitate the comparability of the predictor variables, normalization (variation expressed in terms of interquartile range, or logarithmicization) was performed.

For the Cox regression models, the relative hazard ratio (HR), the 95% confidence interval (CI), the Wald chi-square of the model and its p-value are reported for each unit increase in the predictor variables.

Statistical tests were performed two-sided, with test results considered significant at $p<0.05$.

Statistical calculations were performed with STATISTICA (Statsoft), SPSS (IBM) and GraphPadPrism 4.03 (GraphPad).

4. RESULTS

4.1. Analysis of anaemia and inflammatory markers in relation to heart failure severity

The study included 195 patients with symptoms of heart failure and a left ventricular ejection fraction below 45% on transthoracic echocardiography. The mean follow-up was 14.5 months. The median age of the patients was 69.5 years, the majority of them were male (74.4%), and they tended to be overweight according to body mass index, which could be due to oedema.

The median left ventricular ejection fraction was markedly reduced at 34%. In line with the literature, coronary artery disease was the underlying disease in the majority of cases (62.6%).

Anaemia was found in 21.6% of patients (Hgb ffi≤13 g/L, female≤12 g/L). Patients with anaemia were in 71% and non-anaemic patients in 41% of severe NYHA stages.

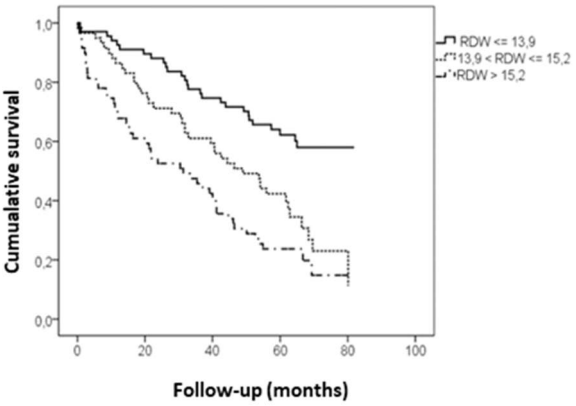
Severe patients (NYHA III-IV) had significantly lower (p0.002) haemoglobin, iron, albumin, transferrin saturation levels and significantly higher ($p<0.018$) CRP, TNF-alpha, soluble transferrin receptor, creatinine, EPO levels and RDW than less severe patients (NYHA I-II). The anaemic group had significantly lower iron, transferrin saturation ($p<0.0001$) and significantly higher soluble transferrin receptor, EPO, CRP, creatinine levels than the non-anaemic group.

4.2. Prognostic role of RDW in heart failure - follow-up data

During the first 12 months of follow-up, 36 patients died and 41 were admitted to hospital for worsening heart failure. We then examined the association of each laboratory parameter with mortality. There was an unexpected and markedly robust result between RDW and mortality. A significant association ($p<0.0001$) was demonstrated between increasing RDW value and heart failure severity and adverse short-term outcome.

Survival curves were defined according to RDW terciles comparing the results of one-year and five-year follow-up. During the 5-year follow-up, 110 patients died (56.4%). Based on Kaplan-Meier survival curves according to RDW terciles, a significant difference in survival between patients in each terciles group was maintained at 5-year follow-up. (1. Figure)

In the multivariate Cox regression models, we examined the predictive value of RDW, when adding NT-proBNP, known as the "gold standard", to the models, RDW remained a strong significant predictor of mortality (HR 1.61, CI 1.302-1.990 $p<0.0001$).



1. **Figure Univariate Kaplan-Meier analysis showing the survival of patient groups based on baseline RDW over the five-year follow-up period.**

4.3. Relationships of RDW with key biological markers in heart failure

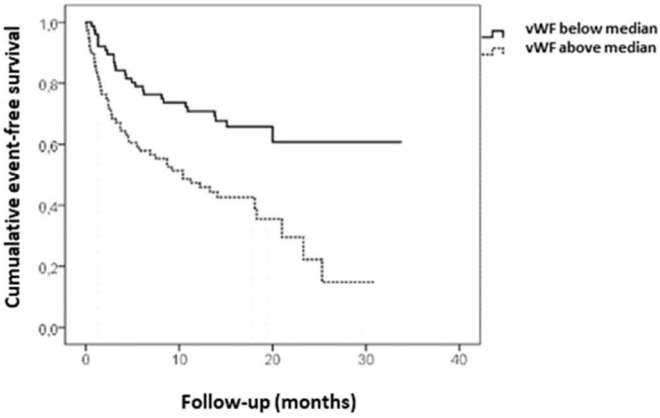
To analyse the biological correlates of RDW and its association with heart failure outcomes, I have chosen biomarkers relevant to the pathophysiological processes underlying heart failure - erythropoiesis, inflammation, renal function, nutrition. The levels of the parameters measured at the time of inclusion were assessed according to the RDW tercilis groups. Patients in the higher RDW group had worse renal function, lower markers of nutritional status and higher inflammatory parameters.

The greatest difference between the lower and upper tertile groups of patients according to RDW was in the measurement of soluble transferrin receptor (2.6x), IL-6 (2.2x) and serum TNF receptor (1.5x) levels. A multivariate linear regression model was used to assess the relative weight and inter-relationship of the associations between each variable and RDW. The variation in RDW was explained by 40-42% of the variables in the table (r2=0.416), with the strongest associations of soluble transferrin receptor, serum TNF receptor and total cholesterol levels with RDW.

4.4. Predictive role of von Willebrand factor (vWF) in heart failure

One early marker of endothelial damage in chronic heart failure is elevated plasma vWF levels.

In a univariate model, higher vWF levels at 1-year follow-up were found to be a significant predictor of cumulative endpoints (death or rehospitalization): HR 3.37 (CI 2.043-5.564, $p<0.0001$, and a significant association was also found at 5-year follow-up (HR 2.68 (CI 1.736-4.151, $p<0.0001$)). (2.Figure)



2. Figure Kaplan-Meier analysis of low and high von Willebrand factor (below and above median) levels and cumulative event-free survival (minimum 5-year follow-up) Log rank test p=0.002

Multivariate Cox regression analysis was used to examine the predictive value of vWF for mortality and rehospitalization after adjustment for key clinical variables such as glomerular filtration rate, heart rate, body mass index, diastolic blood pressure, sodium, hemoglobin level and age. To facilitate comparability, variables were normalized to the interquartile range. vWF remained a significant predictor of one-year mortality or hospital readmission after fitting the NT-proBNP model (HR 2.254, CI 1.180-4.305, p=0.014). The predictive value of vWF remained significant when examining 5-year mortality data using a model with NT-proBNP added to the main clinical variables. (HR 2.463, CI 1.411-4.300, p=0.002)

4.5. The importance of C3a anaphylatoxin in heart failure

In univariate Cox regression analysis, C3a anaphylatoxin has a significant predictive value (HR 1.234 (95% CI 1.044-1.459) for predicting events. Patients were divided into terciles based on C3a level, resulting in 24.6 events per 100 patient-years in the lower third (<185.4 ng/mL), 43 and 54 events per 100 patient-years in the middle (185.4-317.2 ng/mL) and higher thirds (>317.2 ng/mL), respectively.

We used a multivariate Cox model to examine the predictive value of C3a levels for 1-year mortality and hospitalization for heart failure, compared with key clinical characteristics (age, body mass index, diastolic blood pressure, hemoglobin, creatinine levels) and NT-proBNP. The addition of C3a level significantly improved the predictive ability of the model (HR 1.189 Wald $\chi 2$=4.195 and p-value=0.041).

To compare the predictive value of C3a anaphylatoxin level with the predictive value of known biomarkers for events (rehospitalization and death) during one-year follow-up, we first performed a ROC analysis. The area under the curve (AUC) was determined and there were no significant differences for the parameters studied. In multivariable models, we fitted to the main clinical data, diastolic blood pressure, body mass index, age, haemoglobin and creatinine levels, and NT-proBNP, with variables normalised to the interquartile range.

Of the biomarkers tested, only higher C3a anaphylatoxin levels were found to be an independent predictor of mortality and rehospitalization in chronic heart failure independent of NT-proBNP. (1.Table)

1.Table Comparison of the predictive power of C3a anaphylatoxin and inflammatory biomarkers using receiver operating characteristics (ROC) analysis

	AUC	95% CI	Hazard ratio	95% CI
C3a	0,659	0,579-0,739	1,189	1,023-1,383
NT-proBNP	0,692	0,618-0,767	-	-
CRP	0,609	0,530-0,688	0,992	0,795-1,238
Interleukin-6	0,660	0,576-0,744	1,118	0,848-1,473
TNF-alfa	0,592	0,511-0,673	1,164	0,964-1,405
sTNF-RI	0,629	0,549-0,709	0,970	0,808-1,165
sTNF-RII	0,630	0,552-0,708	0,957	0,753-1,215

The area under the ROC curve (AUC) and the 95% confidence interval (CI) are shown

5. CONCLUSIONS

1. Our prospective study included chronic heart failure patients with reduced systolic left ventricular function. Our results show that as chronic heart failure worsens, inflammation increases and anaemia becomes more frequent, with iron deficiency playing a significant role.

2. During 12 months of follow-up, we found that RDW is an independent predictor of mortality or rehospitalization. (HR 1.28, 95% CI 1.066-1.533, p=0.018)
Statistical models suggest that RDW also has significant predictive value independently of the "gold standard" NT-proBNP in predicting mortality in an additive manner. Our group was the first to independently confirm the observation reported by Felker et al. namely the prognostic significance of RDW in chronic heart failure.

3. Based on the literature, I was the first to investigate the relationship between pathophysiological processes involved in the pathogenesis of chronic heart failure and RDW. Significant associations were found between higher RDW and lower GFR ($p<0.0001$), lower cholesterol ($p<0.0001$) and albumin ($p<0.0001$), higher inflammatory biomarkers (IL-6 $p<0.0001$, CRP $p=0.01$) and lower iron ($p<0.001$), as well as higher soluble transferrin saturation ($p<0.0001$).
Statistical analyses confirmed that RDW is an integrating marker associated with all of the above disease processes in the background.

From the results of my thesis, it is worth highlighting that the strongest correlation between RDW and serum soluble transferrin receptor ($r^2=0.339$, $p=0.003$) was found in chronic heart failure.
Based on these findings, RDW appears to be the most prominent marker of anemia associated with chronic disease exacerbated by iron deficiency in heart failure.

4. In heart failure, higher vWF levels are a significant and independent predictor of short- and long-term mortality and rehospitalization, and have additive significant predictive value independently of NT-proBNP in predicting mortality, suggesting a role for endothelial dysfunction in heart failure progression.

5. Higher C3a anaphylatoxin levels in heart failure are a significant and independent predictor of short-term mortality and rehospitalization. Given that it has a significant predictive value independent of NT-proBNP, it can be concluded that complement activation is involved in heart failure progression.

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