Investigating novel and conventional blood biomarkers for diagnosis and prognosis of cardiovascular disorders

PhD Thesis booklet

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1. Prologue

Any characteristics that are measured as indicators of physiological or pathological biological processes or responses to an exposure or intervention can be listed in the group of biomarkers. They can be used for diagnosis, therapy monitoring, measuring pharmacodynamic response, predictive or prognostic purposes, to ensure safety by indicating toxicity or for establishing susceptibility or risk for development of a disease. The ideal biomarker possesses high sensitivity, allowing early detection, and also sufficiently high specificity for a given disease or outcome. It is advantageous if it can be measured easily, inexpensively, and non-invasively producing rapid, reproducible results. Biomarker research should help to better understand underlying pathological processes in a particular medical condition and shed light on new therapeutic perspectives.

My Ph.D. work focused on two main groups of patients with pathological conditions involving the cardiovascular system: patients who suffered cardiac arrest with successful cardiopulmonary resuscitation (CPR) and women diagnosed with early-onset preeclampsia.

Concerning the prognostication of **resuscitated patients**, it is important to have adequate specificity of biomarkers to avoid misjudging an individual with a potential chance to recovery as having a poor prognosis. Since most prognostic tests are focused to predict poor neurological outcome, it is desirable to possess a high specificity, which means a very low rate of falsely pessimistic predictions potentially leading to an inappropriate withdrawal of life-sustaining therapy (WLST). There is no universal consensus on the desired level of specificity of a test for neuroprognostication after cardiac arrest. Requesting 100% specificity would decrease the sensitivity to levels where clinical utility is already equivocal, while allowing a false positive rate of 1-2% would increase the clinical relevance of the biomarker.

On the other hand, in **early-onset preeclampsia**, high sensitivity should be preferred to avoid missing the detection of an individual with potential risk for preeclampsia, even at the cost of more false positive cases, because false negative results are unequivocally more harmful than false positive results. Lower risk threshold and lower positive predictive values may be reasonable for the early detection of an individual with elevated risk for preeclampsia to ensure the opportunity to introduce an early preventive therapy (i.e. low-dose aspirin prophylaxis), guide the surveillance strategy during the pregnancy, and choose the optimal time for delivery.

I. NOVEL AND CONVENTIONAL BIOMARKERS FOR POST-RESUSCITATION PROGNOSIS

2. Introduction

Although the survival of cardiac arrest patients with attempted CPR significantly increased throughout the past decades, the long-term survival remains poor despite all efforts. The mortality after return of spontaneous circulation (ROSC) mostly results from ischaemic brain injury, myocardial dysfunction, multiple organ failure resulting from systemic ischaemia-reperfusion injury and persistent precipitating aetiology. The early death within 3 days occurs mostly due to circulatory failure, while later death is mainly related to severe hypoxic-ischaemic encephalopathy and the subsequent WLST. Death after resuscitated out-of-hospital cardiac arrest (OHCA) occurs mostly due to withdrawal of care because of neurological reason, while this occurs less than one third of cases after in-hospital cardiac arrest (IHCA), where underlying comorbidities, refractory hemodynamic shock, and multiple organ failure drive mortality.

Early predictors of outcome that would support clinical decision-making are required to avoid inappropriate WLST or costly, prolonged treatment in patients with no chance of neurologically meaningful survival and to correctly guide goals-of-care conversations with family members. Current guidelines recommend a multimodal approach to assess the severity of hypoxic-ischaemic brain injury combining multiple methods including biomarkers to reduce the risk of falsely pessimistic prediction. It is challenging to find a reliable biomarker for identifying patients with poor outcome due to the variability of measurement techniques and thresholds. In addition, the sometimes-limited availability, and the weak evidence due to small sample size limit the general usability of biomarkers. Moreover, it is difficult to determine the proper and consistent cut-off with maximal specificity and acceptable sensitivity for poor outcome. On the other hand, biomarkers have many advantages: they provide quantitative information, not affected by the presence of sedation or paralysis; moreover, they are easy to measure with an appropriate laboratory background and can be evaluated blindly to other clinical data excluding subjective prophecy about outcome of the patient. Consequently, the investigation of traditional biomarkers from new aspects and the evaluation of novel biomarkers followed by their incorporation in prognostic algorithms are certainly justified.

Current recommendations are mostly suitable for neurological prognostication of unresponsive, comatose patients, and although high proportion of patients reach acceptable neurological function, they may die independently of neurological status due to their underlying comorbid conditions, haemodynamic instability or later-developing multiple organ failure. Therefore, it would be important to broaden the prognostication strategy after cardiac arrest and reconsider whether the general conception to evaluate biomarkers reflecting exclusively neurological injury is correct. It is worth investigating and finding biomarkers with potential additional information about overall survival to complete the current prognostication algorithm. Identification of reliable indicators is essential to predict overall outcome, thereby improving understanding of the aetiology, and to guide post-resuscitation management.

2.1. L-arginine pathway molecules

Cardiac arrest leads to endothelial dysfunction, which can play a potentially important role in the development of post-cardiac arrest syndrome. Therefore, endothelial injury and subsequent microcirculatory dysfunction are associated with poor outcome of resuscitated patients. Impaired nitric oxide synthesis is considered a major feature of a dysfunctional endothelium. Nitric oxide, a pleiotropic molecule, has several intracellular effects leading to vasorelaxation, endothelial regeneration, inhibition of leukocyte chemotaxis, and platelet adhesion. L-arginine pathway molecules are one of the main regulators of nitric oxide synthesis and vascular regulation, hence indicators of endothelial dysfunction. L-arginine is a substrate for nitric oxide synthase, which catalyses its two-step oxidation to nitric oxide and L-citrulline in endothelial cells, thus regulating vascular tone and cardiovascular homeostasis. Methylarginines are the main regulators and endogenous inhibitors of nitric oxide synthase catalytic function. Asymmetric dimethylarginine (ADMA) is a direct competitor for binding to the catalytic site of nitric oxide synthase, in addition ADMA and symmetric dimethylarginine (SDMA) compete with L-arginine at the level of transport into the cell as well. ADMA has been previously described to inhibit nitric oxide formation and increase oxidative stress in vascular endothelial and smooth muscle cells. The bioavailability of nitric oxide depends on the balance between L-arginine and ADMA, consequently the reduced L-arginine/ADMA ratio results in the inhibition of nitric oxide production. Increased ADMA levels were observed in hypertension, hypercholesterolemia, diabetes, and atherosclerosis, and the elevated levels are associated with progression and outcome in several cardio- and cerebrovascular disorders and with mortality of critically ill and septic patients.

2.2. Cell death and cytokeratins

Cytokeratins are cytoskeletal structural proteins and members of intermediate filament superfamily in epithelial and parenchymal cells. As the consequence of cardiac arrest and ischaemic-reperfusion conditions the systemic cell damage and subsequent apoptotic and necrotic cell death is amplified. During apoptosis caspases cause the fragmentation of the cytokeratin-18 (CK-18), forming caspase-cleaved cytokeratin-18 (CCCK-18), which hence is considered to be an apoptosis-specific cell death biomarker. On the other hand, necrotic cell death results in the release of the full-length CK-18 to the circulation. The fragments of the CK-18 cleaved by caspases can be recognised by a monoclonal antibody and in combination with the full CK-18 measurement, the predominant mode of cell death can be determined using the CCCK-18/CK-18 ratio. The lower this ratio, the more necrosis dominates the cell death processes. The increased level of CCCK-18 in septic and critically ill patients was associated with mortality in previous studies.

3. Hypothesis and objectives

We aimed to identify potentially promising biomarkers in the early post-resuscitation phase, which could provide additional information about the overall survival of unselected resuscitated patients. Systemic endothelial injury and cell damage are presumably amplified as the consequence of ischaemic reperfusion injury after resuscitation. Therefore, we focused on investigating markers reflecting these pathological phenomena. L-arginine, ADMA, SDMA, and CK-18 and its caspase-cleaved form were described earlier as potential prognostic markers

in various acute and chronic cardio- and cerebrovascular disorders and critical illness. Although they have not been evaluated yet among unselected resuscitated patients, we assumed that the circulating levels may associate with the outcome.

- The primary objective of our study was to investigate the peripheral blood concentrations of the above-mentioned markers and their kinetics with repeated sampling in the early post-resuscitation care and characterise them according to survival.
- Our secondary objectives were to evaluate their distinct association patterns with conventionally used prognostic scoring systems (Simplified Acute Physiology Score - SAPS II - and Sequential Organ Failure Assessment - SOFA) and with the characteristics and circumstances of cardiac arrest and CPR. Furthermore, we aimed to reveal if there is any impact of neurological status on the marker levels.
- Besides identifying novel biomarkers, we aimed to determine neuron-specific enolase (NSE) levels, conventionally used laboratory, clinical and vital parameters according to survival and neurological outcome and to test the prognostic accuracy of SAPS II and SOFA.

4. Materials and methods

4.1. Study design, subjects

We conducted a prospective, single-centre observational cohort study adhering the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) Statement, from January 2018 to January 2019 in the Intensive care unit of the 1st Department of Medicine, Department of Anaesthesiology and Intensive Care and Department of Emergency Medicine at the University of Pécs. We enrolled adult (age ≥18 years) patients after successful CPR admitted to the ICU for post-resuscitation care regardless of the aetiology, initial rhythm, or whether it was in- or out-of-hospital. Successful resuscitation was defined as the return of spontaneous circulation (ROSC). Standard post-resuscitation care was applied for each patient in the ICU without interaction with the research team. Therapeutic hypothermia was not applied; however, the overall goal was to maintain normothermia and to prevent fever. The total follow-up period was 30 days after cardiac arrest. The study was approved by the Local Ethics Committee of the University of Pécs (6941 – PTE 2018.) and followed the principles of the Declaration of Helsinki. Informed consent for being included in the study was obtained from patients or legal representatives.

4.2. Data collection

We collected general information about patients (e.g. age, gender and comorbid conditions). Prognostic scores (SOFA and SAPS II) were calculated according to the worst parameters of the first 24 hours after cardiac arrest using an online calculator (https://www.mdcalc.com/). We recorded the presumed cause of cardiac arrest and categorised them into groups according to the most common aetiological factors occurred in our cohort (ischaemic heart disease, heart failure; sepsis; hyperkalaemia; aspiration; hypothermia; stroke; pulmonary embolism; pneumonia or unknown). The circumstances of CPR were also reported (e.g. localisation of cardiac arrest; first monitored rhythm; length of the CPR; epinephrine requirement and dose; mechanical ventilation requirement). We documented the most important vital parameters at enrolment (systolic and diastolic blood pressure; mean arterial

pressure; heart rate and body temperature). Conventionally measured laboratory parameters required for routine post-resuscitation care were also collected (e.g. electrolytes; markers of renal and hepatic function; inflammatory parameters; troponin-T, lactic dehydrogenase; complete blood count; lactic acid; blood gas parameters). Furthermore, we examined the presence of vital organ system failure during the post-resuscitation period (i.e. circulation, respiration, liver and kidney function).

4.3. Sample collection and processing

Blood samples were drawn from routinely provided arterial or central venous cannula into Vacutainer® EDTA-tubes within 6, at 24±3 and 72±3 hours after cardiac arrest. The blood samples were centrifuged within 10 minutes at 1500 g for 15 minutes. The plasma supernatant was immediately portioned out into cryo tubes and stored at −80 °C until processing. Plasma concentrations of CK-18, CCCK-18, and NSE were determined in collaboration with the Department of Laboratory Medicine (University of Pécs, Hungary) by using enzyme-linked immunosorbent assay kit (CCCK-18, CK-18 - Shanghai YL Biotech Co., Ltd., China; NSE - FineTest, Wuhan Fine Biotech Co., Ltd., China) with the detection limit of 5.64 ng/L, 19.00 ng/L and 1.41 ng/mL, respectively. The CK-18 assay detects both intact and cleaved fragments, thus it refers to total cell death, while the CCCK-18 assay binds only the cleaved variant thus indicating only apoptosis. L-arginine, ADMA, and SDMA were measured by high-performance liquid chromatography after derivatisation in collaboration with the Department of Applied Chemistry (University of Debrecen, Hungary). We calculated the change of the investigated markers from 6 to 24 and 24 to 72 post-cardiac arrest hours. At each time point, derived parameters were calculated: CCCK-18/CK-18 ratio to establish the dominant mode of cell death and L-arginine/ADMA ratio reflecting the nitric oxide production. All samples were processed by the same technicians using the same equipment and blinded to all clinical data. The biomarker values were blinded to clinicians to avoid the influence on post-resuscitation care approaches or decision-making processes.

4.4. Outcomes

As the follow-up period lasted until 30 days after cardiac arrest, we determined three different mortality endpoints. The primary outcomes of the study included mortality within 72 post-cardiac arrest hours, during ICU stay and within 30 days. Besides, we determined SOFA and SAPS II, the presence of different vital organ system failure (circulatory, respiratory, liver, kidney) and neurological status as secondary outcomes. The neurological condition was described according to Cerebral Performance Category (CPC) score. CPC 1 means intact brain function or minimal brain injury, CPC 2 includes patients with minor neurological disabilities, CPC 3 implies a wide range of different severe neurological disabilities, CPC 4 indicates persistent vegetative state, while CPC 5 is regarded as death or brain death. The best neurological status reached in the ICU stay was recorded and dichotomised according to good (CPC 1-3) and poor (CPC 4-5) neurological outcome.

4.5. Statistical analysis

Kolmogorov-Smirnov test was used to assess normality. Variables are expressed as mean ± standard deviation, or as median with interquartile range, or as frequencies and

percentages. Mann-Whitney U-test, Student T-test or Chi-square test were applied for comparison of data between groups. Correlation analysis was performed calculating Spearman's correlation coefficient (rho). For variables with significant correlation, linear logistic regression analysis was also performed, and R^2 values were reported. Receiver operating characteristic (ROC) analysis and the area under the curve (AUC) were used to determine the most appropriate cut-off values of investigated biomarker levels for study endpoints, "z" tests were used for comparison of multiple ROC curves. Univariate binary logistic regression tests were used to evaluate association between the recorded initial variables and mortality displaying the corresponding beta values and 95% confidence intervals. Variables with $p \leq 0.05$ in the univariate analysis were included in the multivariable models considering the principle of multicollinearity. Multivariable logistic regression was used to identify factors independently associated with mortality. IBM SPSS Statistics® 27.0 software was used for statistical analysis of the collected data. $P<0.05$ was considered statistically significant.

5. Results

5.1. Characteristics of the study cohort

A total of 54 patients were enrolled (median age: 67 [61-78] years, 48% male); 72% were IHCA. Half of the patients reached acceptable neurological status (CPC 1-3) during the ICU stay, while the other half suffered from coma, persistent vegetative state, or brain death (CPC 4-5). 8 patients who reached acceptable neurological function (CPC 1-3) later died due to non-neurological reasons in the ICU. *Figure 1.* shows the flow chart about the exact numbers of survivors at each investigated endpoint. The characteristics of survivors and non-survivors according to 30-day, ICU and 72-hour mortality are shown in tables of the full thesis (*Table 3.* and *Appendix 1-2.*).

Among 30-day survivors, the CPC category was significantly better (3.0 [2.3 – 3.0] vs. 4.5 [3.0 – 5.0]; $p < 0.001$), as expected and significantly more favourable SAPS II and SOFA scores were recorded. The age, gender and the length of the CPR, initial rhythm did not influence the survival at any investigated endpoint, and mortality was independent of whether the cardiac arrest occurred in- or out-of-hospital or during working hours or nightshift/weekend. The mechanical ventilation and epinephrine requirement and the basic vital parameters on enrolment were also similar between survivors and non-survivors. The percentage distribution of the comorbidities, past medical history and presumed cause of cardiac arrest also showed no relevant significant difference between survivors and non-survivors.

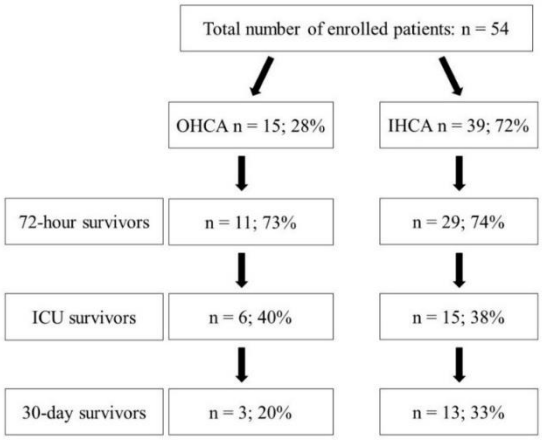

Figure 1. *Number and percentage of survivors at 72 hours, at ICU discharge, and 30 days after cardiac arrest*

5.2. L-arginine, ADMA, SDMA

We investigated the absolute plasma levels of L-arginine, ADMA, and SDMA and their change over the first three post-cardiac arrest days between **72-hour** survivors and non-survivors (*Table 4.* in the full thesis). Significantly higher initial ADMA levels were observed among patients who died within 72 hours after cardiac arrest (0.55 [0.45 – 0.69] μmol/L vs. 0.88 [0.64 – 0.97] μmol/L, p=0.001). We did not observe significant difference in initial ADMA levels according to the location of CPR.

Investigating the **ICU mortality**, none of the L-arginine pathway molecules showed significant difference between survivors or non-survivors. The initial ADMA levels tended to remain higher among ICU non-survivors, but the difference did not reach significance. The plasma ADMA levels of ICU non-survivors decreased from 6 to 24 hours, while the values of the surviving group raised (-0.08 [-0.16 – 0.05] μmol/L vs. 0.07 [-0,04 – 0.11] μmol/L, p= 0.024) (*Table 5.* in the full thesis). Subgroup analysis of IHCA patients revealed significantly decreased 6-hour L-arginine/ADMA ratio in ICU non-survivors (52.16 [34.96 – 71.99] vs. 73.43 [51.24 – 98.56]; p=0.023) (*Figure 6.* in the full thesis).

Analysing the kinetics of the markers according to **30-day mortality**, an opposite change was observed in ADMA level from 6 to 24 hours between the groups (-0.08 [-0.16 – 0.06] μmol/L in non-survivors vs. 0.07 [-0.03 – 0.11] μmol/L in survivors, p=0.028) similarly to the observation according to ICU mortality (*Table 6.* in the full thesis). In contrast, L-arginine, SDMA levels, or their change showed no significant difference at any investigated time point. The L-arginine/ADMA ratio slightly elevated up to 72 post-cardiac arrest hours in the total population regardless of the mortality (6 h: 66.04±4.33; 24 h: 80.04±5.35; 72 h: 99.99±7.13; $p<0.05$) (*Figure 7.* in the full thesis).

Neither SAPS II nor SOFA score showed significant difference between IHCA and OHCA subgroups. The statistical analysis revealed a significant positive correlation between the initial ADMA levels and the SAPS II score (rho=0.393, R^2=0.178, p=0.002) (*Figure 8.* in the full thesis).

Significantly elevated initial ADMA levels were detected among patients with persistent vegetative state or brain death (CPC 4-5) (*Figure 2.*). *Figure 3.* demonstrates the curves of combined ROC analysis of SOFA, SAPS II, and initial ADMA for 72-hour mortality and poor neurological outcome (CPC 4-5). The results showed that the AUC of SAPS II and initial ADMA were comparable reflecting similar sensitivity and specificity in prediction of 72-hour mortality, in contrast SOFA provided poor prognostic information for mortality (SAPS II AUC: 0.817 [0.688 – 0.946], p<0.001; ADMA AUC: 0.789 [0.628 – 0.950], p=0.001; SOFA AUC: 0.608 [0.433 – 0.783], p=0.232).

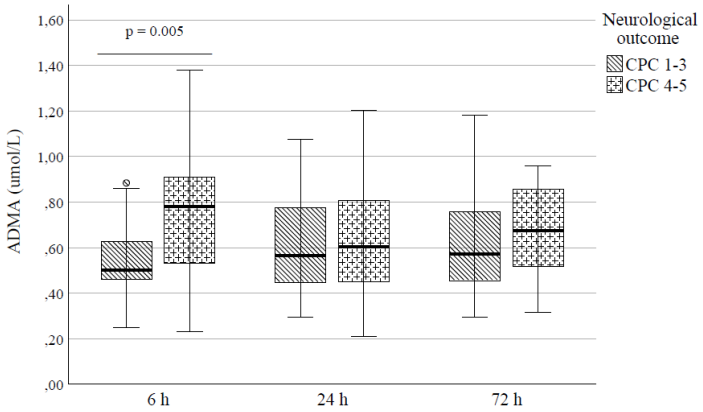

Figure 2. *ADMA levels according to acceptable (CPC 1-3) and poor (CPC 4-5) neurological category*

ROC analysis of initial ADMA for prediction of poor neurological outcome (CPC 4-5) showed an AUC of 0.723 [0.574 – 0.871] (p= 0.005). Based on the ROC analysis, the best cut-off for poor neurological outcome (CPC 4-5) was determined as >0.65 µmol/L (sensitivity: 66.7%; specificity: 81.5%). The values over 0.89 µmol/L have maximal specificity (100%) for CPC 4-5 with 33.3% sensitivity.

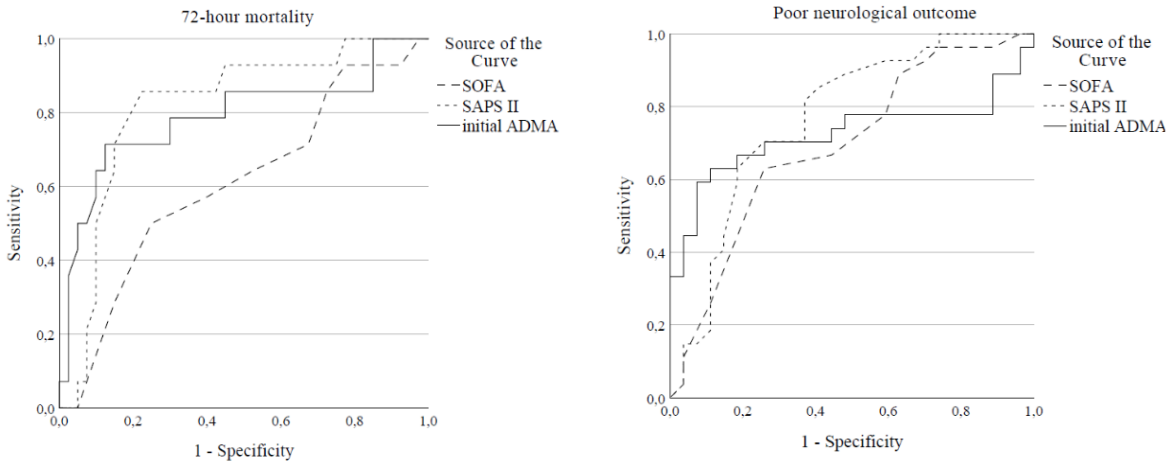

Figure 3. *ROC Curves of initial ADMA, SOFA, and SAPS II for 72-hour mortality and poor neurological outcome*

Based on ROC analysis, initial ADMA level was found to be a predictor of 72-hour mortality with a best cut-off value of >0.81 µmol/L (sensitivity: 71.0%; specificity: 87.5%). Univariate logistic regression analyses including each variable assessed within 6 hours after cardiac arrest identified initial ADMA, serum bicarbonate (HCO_3^-), and lactate levels as significant markers for 72-hour mortality. Multivariable analysis revealed that initial ADMA

(OR: 1.8 per 0.1 µmol/L increase in ADMA; 95% CI: 1.252 – 2.611; p=0.002) is an independent predictor for 72-hour mortality after cardiac arrest (*Table 7.* in the full thesis).

5.3. Markers of cell damage and death - CK-18, CCCK-18 and NSE

There was no significant difference regarding absolute levels or kinetics between survivors and non-survivors according to 72-hour, ICU or 30-day mortality for any of the investigated markers discussed in this subsection. We did not observe significant change in the marker levels over the first three days in any patient group. We could not confirm connection between the cell death marker levels and neurological outcome either. Although the initial and 24-hour CK-18 values were not associated with the prognostic scores, the 72-hour CK-18 level showed significant correlation with SAPS II (rho=0.581; p< 0.001) and SOFA scores (rho=0.418; p=0.012). The results of linear regression analyses are illustrated in *Figure 4*. We did not observe this connection concerning CCCK-18, the marker of apoptosis.

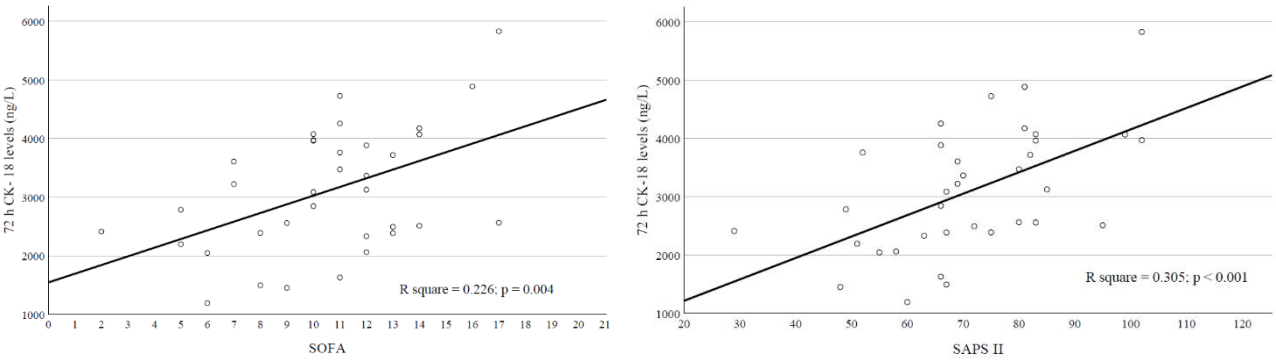

Figure 4. *Linear regression analysis of 72 h CK-18 levels with SOFA and SAPS II score*

Our results show the lack of any association between the number of organ system failures and the extent of cell death reflected by cytokeratins. On the other hand, subgroup analysis revealed that the CK-18 level did not decrease over the first three days after ROSC in the presence of renal failure compared to patients with intact renal function, where a decreasing kinetic was visible, resulting in significant difference at 72 hours (*Figure 5.*).

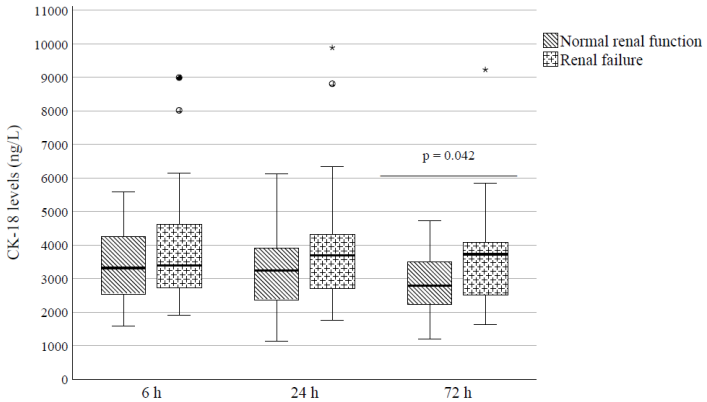

Figure 5. *CK-18 levels according to normal or impaired renal function*

To better understand which factors influence the circulating concentration of CK-18 and CCCK-18 cell death markers after cardiac arrest, we performed subgroup analyses concerning different aspects of CPR characteristics. According to our results, the location of cardiac arrest (IHCA or OHCA) or the length of the CPR, or the initial rhythm were not associated with the

circulating marker levels at any investigated time points over the first three days after ROSC. Figures of these non-significant results are illustrated in the full thesis (*Figures 11-20*).

5.4. Laboratory parameters – lactate

We recorded the conventionally used laboratory parameters in the first three days analysed according to 30-day mortality after cardiac arrest. The values are summarised in detail in the full thesis *(Table 8)*. Initial and 24-hour lactate levels were higher among non-survivors (initial: 3.2 [2.1 – 4.6] vs. 7.1 [4.3 – 9.7] mmol/L; p=0.005; and 24 h: 1.0 [0.8 – 1.5] vs. 1.7 [1.1 – 2.5] mmol/L; p=0.008) as well and patients with higher initial lactate (3.6 [2.1 – 5.7] vs. 8.7 [6.0 – 10.7] mmol/L; p<0.001) or 24-hour lactate levels (1.1 [0.9 – 1.7] vs. 1.8 [1.1 – 2.5] mmol/L; p=0.030) were more prone to have poor (CPC 4-5) neurological status (*Figure 6.*)

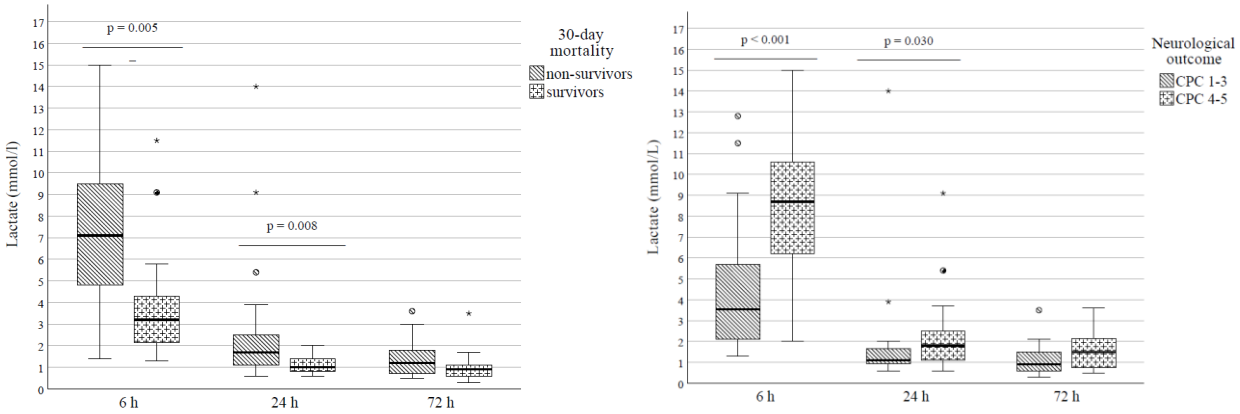

Figure 6. *Lactate levels at the investigated time points according to 30-day mortality and neurological category (CPC 1-3 as acceptable vs. CPC 4-5 as poor outcome)*

CK-18, CCCK-18, NSE or their change were not found to be significantly related to 30-day mortality with univariate regression analysis. However, initial lactate seemed to predict 30-day mortality and neurological outcome, while 24- and 72-hour lactate values had mild importance compared to initial lactate levels, therefore, in later statistical analyses we considered initial lactate.

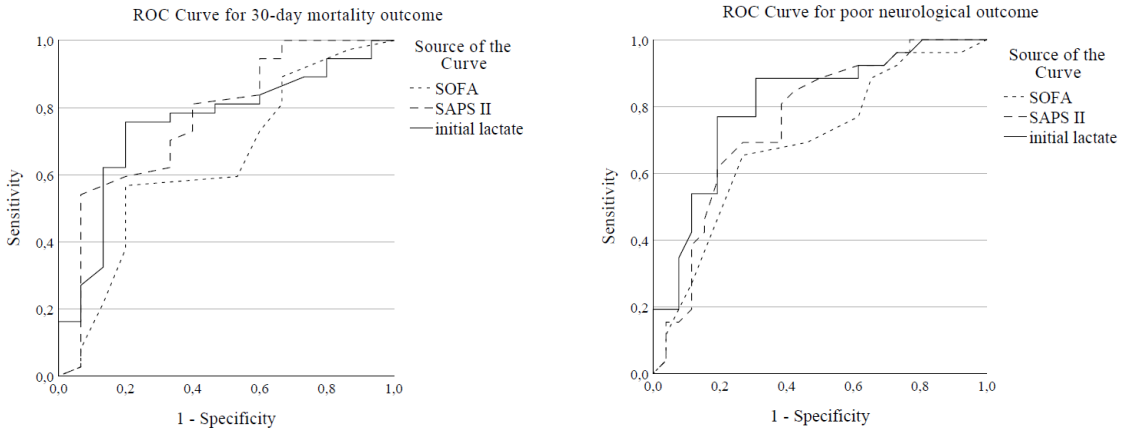

Figure 7. *ROC Curve of initial lactate, SAPS II and SOFA for 30-day mortality and for poor neurological outcome (CPC 4-5)*

ROC analysis for prediction of the 30-day mortality was carried out with SOFA, SAPS II, and lactate levels. AUC values were 0.638 [0.465 – 0.811] (p=0.122) for SOFA, 0.767 [0.616 – 0.917] (p=0.003) for SAPS II and 0.753 [0.607 – 0.899] (p=0.005) for lactate, respectively. In mortality prediction, SOFA had poor, SAPS II had moderate value, while the AUC of lactate per se was similar to SAPS II based on ROC analysis. There was no statistically significant difference comparing the AUC values (SAPS II vs. lactate p=0.892; SAPS II vs. SOFA p=0.088; lactate vs. SOFA p=0.373). (*Figure 7.*)

ROC analysis was performed with the same variables for prediction of poor neurological outcome defined as CPC 4-5. AUC values were 0.689 [0.544 – 0.835] (p=0.019) for SOFA, 0.757 [0.623 – 0.891] (p=0.001) for SAPSII and 0.806 [0.685 – 0.928] (p<0.001) for lactate. As for poor neurological outcome, lactate per se had good predictive value, however, the differences of the AUC values were not statistically significant (SAPS II vs. lactate p=0.356; SAPS II vs. SOFA p=0.322; lactate vs. SOFA p=0.129). The ROC curve of initial lactate per se indicated a cut-off as 4.90 mmol/L to predict 30-day mortality (sensitivity: 74%; specificity: 80%) and as 6.00 mmol/L (sensitivity: 84%, specificity: 80%) for poor neurological outcome (CPC4-5).

6. Discussion

6.1. Summary of findings

Our main goal was to assess early biomarkers with potential prognostic value for overall survival in general cohort of resuscitated patients. We investigated two main groups of biomarkers, the L-arginine, ADMA and SDMA line reflecting impaired endothelial function and vascular regulation and CK-18 and its caspase-cleaved form referring indirectly to cell death as the consequence of ischaemic insult. As to date no data is yet available concerning these biomarkers among unselected resuscitated patients, we evaluated the absolute value and kinetics of these markers from repeated sampling on the first three days after ROSC to find the most suitable time where these parameters would have the highest prognostic value. Besides, we investigated conventionally used laboratory parameters, prognostic scoring systems and previously well-investigated NSE. Our results suggest that initial circulating ADMA may indicate more severe hypoxic insult and can predict short-term 72-hour mortality. However, a clear connection was missing between L-arginine pathway molecules and death in later post-resuscitation period. Although the 72-hour CK-18 values showed a clear correlation with the recorded SAPS II and SOFA scores, we could not prove the prognostic value of these cell death markers for mortality or neurological outcome. Moreover, NSE was not a useful predictor of survival or neurological outcome in our cohort either. In line with previous literature, elevated initial lactate was found to be a promising predictor for 30-day mortality and poor neurological outcome. We would like to emphasise the need to develop a generally applicable prognostic algorithm with integration of novel or known biomarkers that can help predict overall survival in resuscitated patients including those individuals for whom limited data are available about the circumstances of cardiac arrest and resuscitation.

6.2. L-arginine pathway molecules

One of our most important observations is that elevated initial ADMA independently predicted short-term mortality and it was associated with poor neurological outcome. However, L-arginine, SDMA, L-arginine/ADMA ratio and their kinetics did not prove to be useful predictors. ADMA is a known prognostic marker of several cardiovascular diseases, hence it may be a rational observation that ADMA predicts early death during post-resuscitation care, as it occurs rather due to cardiovascular failure and haemodynamical instability.

L-arginine pathway molecules have been investigated in various hypoxic conditions, where elevated ADMA levels were related to disease severity or exacerbation. Elevated plasma ADMA levels were detected after acute ischaemic stroke and rising concentrations were associated with worse outcome. The production of endothelial nitric oxide may be attenuated by ADMA excess, which also enhances the arterial stiffness and tone in cerebral blood vessels, consequently leading to cerebral hypoperfusion. Elevated initial ADMA after ischaemic stroke could be linked to the pathogenesis of endothelial cell dysfunction or could be the consequence of oxidative stress. ADMA per se might contribute to brain injury by facilitating excitotoxic neuronal death as well. Therefore, excessively high initial ADMA may adversely affect cerebral perfusion after cardiac arrest, leading in short term to exacerbation of hypoxic-ischemic injury. On the other hand, elevated initial ADMA levels might indicate a more severe hypoxic insult or pre-existing endothelial dysfunction. Normally, the mean plasma nitric oxide concentration should upgrade after acute hypoxic exposure as a consequence of diminished circulating ADMA levels. Conversely, we observed a mild decrease in ADMA levels among non-survivors, while in survivors the values slightly increased by 24 hours. This finding suggests that the decrease in ADMA concentration observed on the first day after cardiac arrest in our more severe group of patients who died within 30 days may be an adaptive mechanism, presumably an attempt to counteract cerebral hypoperfusion by restriction of ADMA excess.

Lack of L-arginine, the source of nitric oxide, could lead to more severe oxidative stress induced by hypoxic insults, which may explain that the group of IHCA patients with diminished initial L-arginine/ADMA ratio were more prone to die during ICU stay. In a recent study, higher arginine and lower arginine/ADMA ratio were proved to be independently associated with mortality after OHCA, while ADMA alterations were unremarkable. The pathophysiological background and aetiology responsible for mortality during post-resuscitation care may differ in our cohort comprised mostly IHCA patients, where multiple organ failure drives the mortality instead of hypoxic brain injury.

6.3. Cell death markers: cytokeratins and NSE

To the best of our knowledge, CK-18 and its caspase-cleaved form has not yet been evaluated among cardiac arrest victims. CK-18 levels reflecting total cell death were persistently elevated accompanied by decreased CCCK-18/CK-18 ratio on the first three days of post-resuscitation care, compared to populations of other studies that refer to a large extent of cell death dominantly due to necrosis. Contrary to our expectations, survival was not associated with the concentrations and kinetics of CK-18, CCCK-18, or CCCK-18/CK-18 ratio. We could not prove any relevant associations analysing these markers according to the CPR

characteristics or the presence of organ failures apart from the elevated CK-18 levels in renal failure, which latter finding is consistent with previous investigations and could be due to the impaired renal elimination and less probably due to the increased release from injured renal epithelial cells.

Considering the above-mentioned observations, we suggested that mortality may rather be determined by the damage of a smaller group of cells responsible for critical function and survival, but this signal may vanish in the mass of total cell death. Survival may rather depend on the remaining functional capacity and the ability to recover than the extent of damage that the above-mentioned biomarkers indirectly represent.

Cytokeratins appear mostly in epithelial cells but not in neurons, where intermediate filaments are made up of neurofilaments, thus peripheral blood levels are not specific to neuronal cell death, which could be the reason why these markers were independent from neurological status. Consequently, we evaluated NSE representing neuronal injury, which was described as a prognostic marker for poor neurological outcome after cardiac arrest. In contrast to our expectations, there was no significant difference in our study population concerning this marker and we could not confirm the prognostic value for mortality or neurological outcome. Explanation could be the high heterogeneity of our unselected population, which was mostly composed of various IHCA cases, where NSE has less accuracy due to the higher number of confounders and mortality without hypoxic-ischemic encephalopathy and even the OHCA group had diverse aetiology.

6.4. Lactate

Failure of tissue perfusion during cardiac arrest leads to anaerobic metabolism. Lactate is the end-product of anaerobic metabolism that can be used as a marker of cellular hypoxia and to predict mortality in critical illness. Previous investigations have proved the utility of lactate as a marker for disturbances of tissue perfusion to predict survival in cardiac arrest patients. We confirmed the prognostic role of elevated initial lactate levels for 30-day mortality (>4.90 mmol/L) and poor neurological outcome (>6.00 mmol/L). Similar lactate cut-off values were reported previously and thought to be associated with outcomes at discharge from the ICU after cardiac arrest, thus lactate was suggested as a promising predictor especially after IHCA. According to our results elevated lactate levels measured within 6 hours after cardiac arrest could help the prediction of coma, vegetative state, or brain death.

6.5. SOFA, SAPS II, and biomarkers

The prognostic reliability of SOFA and SAPS is equivocal due to their moderate discrimination ability among cardiac arrest victims. Therefore, significant efforts have been made in recent years to develop more specific scoring systems for the outcome estimation of resuscitated patients. Despite being promising, these new scores are not as widely used as classical general prognostic scores and often require missing background information about the patient or peri-arrest circumstances, so we chose conventionally used SOFA and SAPS II as outcome measures in our study. We confirmed the association of SOFA and SAPS II with L-arginine pathway molecules, which observation is consistent with previous research findings among septic and critically ill patients. Based on our findings the prognostic accuracy of SAPS

II (a time-consuming assessment method) and initial ADMA for 72-hour mortality after ROSC were comparable. We conclude that early determination of initial ADMA after ROSC may be as effective and accurate as SAPS II in prediction of the early post-CPR mortality. Regarding the later post-resuscitation mortality prediction, initial lactate values per se had similar moderate prognostic value as SOFA and SAPS II for 30-day mortality.

72-hour CK-18 values significantly correlated with both SAPS II and SOFA score, suggesting that systemic cell death processes are connected to overall functional capacity and organ function abnormalities represented by these scores. However, the overall outcome did not associate with cell death markers. On the other hand, the apoptosis-specific CCCK-18 did not associate with prognostic scoring systems.

6.6. Strengths, limitations and future perspectives

The strength of our study is its prospective nature using serial sampling to evaluate the kinetics and changes of multiple conventional and novel biomarkers in unselected resuscitated patients to explore reliable predictors regardless of the circumstances and aetiology of cardiac arrest. While most studies focused on the neuroprognostication of OHCA patients, we were able to find potential prognostic markers for overall outcome in IHCA patients and markers which might be also used in both groups. Although patients reach acceptable neurological function during their post-resuscitation care, they may later die as the consequence of multiple organ failure. Therefore, the neurological outcome and the overall outcome were separately analysed, and the best achieved CPC score was recorded during the ICU stay. The main limitation of our study is the low total number of enrolled patients. Concerning the high mortality rate, we could not collect enough data for long-term analysis. The study was conducted in a single centre, so local treatment strategies and guidelines could limit the generalizability of our findings.

Further multicentre studies with large numbers of subjects allowing long-term follow-up are needed to confirm the prognostic significance of L-arginine pathway molecules in a later (e.g. rehabilitation) phase of post-cardiac arrest patients. This contributes to better understanding the pathophysiological processes in the post-resuscitation period, thus these molecules may not only have a prognostic role but may also open up new therapeutic approaches. Recently, there has been a growing interest in the potential therapeutic effects of arginine supplementation in cardiovascular disorders and critically ill patients. Administration of external arginine or the suppression of arginase enzyme may improve the production of nitric oxide by optimization intracellular L-arginine bioavailability and balancing the arginine/ADMA ratio. Elevated ADMA levels and increased ADMA/arginine ratio may help to select those individuals who could potentially benefit from supplementation.

II. MATERNAL HEMORHEOLOGICAL PROPERTIES IN EARLY-ONSET PREECLAMPSIA

7. Introduction

Preeclampsia is one of the leading causes of maternal and perinatal morbidity and mortality affecting 5-7% of pregnancies worldwide and being responsible for over 70 000 maternal and 500 000 foetal deaths worldwide every year. It is defined as gestational hypertension in previously normotensive women accompanied by new-onset proteinuria and/or maternal organ and/or uteroplacental dysfunction manifesting at or after 20 weeks of gestation. Early-onset preeclampsia develops before 34 weeks of gestation and has higher risks of maternal morbidity, perinatal death and severe neonatal morbidity compared to the late onset form (≥ 34 weeks of gestation).

The pathophysiological explanation of early-onset preeclampsia is predominantly a defective placentation during the first few weeks of pregnancy, but the exact processes in the background are still equivocal. It is suggested that impaired cytotrophoblast invasion into spiral arteries of the placenta in the first trimester result in abnormal vascular remodelling with resistant vessels and elevated pressure. The placental hypoxia leads to the release of pro-inflammatory factors which might contribute to a pathological systemic endothelial response, characterised by increased capillary permeability, microvascular thrombosis, and sustained vascular hypertension.

Short-term maternal consequences could be life-threatening conditions such as placental abruption, acute pulmonary oedema, respiratory distress syndrome, acute renal failure, stroke, eclampsia, multiple organ failure, and disseminated intravascular coagulation. The leading cause of maternal death is cerebral haemorrhage, which is presumably a consequence of severe hypertension. Women with a history of preeclampsia are prone to suffer from cardiovascular disease and have an increased risk for myocardial infarction, heart failure, hypertension, or stroke in later life. The consequences affect not only the pregnant women but can be lethal for the foetus as well, since the placenta is not able to ensure adequate perfusion resulting in prematurity, intrauterine growth retardation or death. Prematurity itself can lead to serious complications both in the perinatal period and in later childhood, consequently affecting the health of the new-born for the rest of its life. Preterm birth is associated with higher rates of infant respiratory distress syndrome, intraventricular haemorrhage, sepsis, bronchopulmonary dysplasia, and later neurodevelopmental disability in childhood.

7.1. Preeclampsia and hemorheology

The science of hemorheology investigates blood flow conditions, the physical properties of blood elements, including cellular and plasmatic components. Deterioration of these factors leads to impaired microcirculation and tissue perfusion. Red blood cell (RBC) aggregation means the rouleaux formation of RBCs under low flow conditions. Its increase enhances friction between fluid plates, deteriorates the blood viscosity, thus adversely affecting both macro- and microcirculation. Erythrocyte deformability is described as the ability of RBCs to adapt by deformation in response to mechanical forces to facilitate crossing over narrow capillaries and ensuring sufficient tissue oxygenation. Rigid RBCs with reduced deformability are unable to

adapt to shear forces, thus higher viscosity values can be measured, especially when higher shear stresses are applied. The elevated RBC aggregation and decreased deformability can deteriorate the tissue perfusion.

Pathologic alterations in blood rheology and impaired blood flow at the uteroplacental cross-over were already previously considered as possible triggers or consequences of preeclampsia. Elevated RBC aggregation and decreased deformability were observed and suggested to be associated with the presence of foetal growth restriction. However, others could not confirm alterations of RBC properties in preeclampsia. Limited information is available about the peripartum and postpartum period in the early-onset form, since previous investigations usually did not distinguish between early- and late-onset forms, although it is nowadays an increasingly accepted fact that the pathophysiological background of the two forms may be different. Therefore, further investigations are required to reveal the pathophysiological and prognostic significance of these parameters, especially in early-onset form, which has more severe short- and long-term maternal and foetal consequences.

8. Hypothesis and objectives

Our goals were to expand the knowledge about the maternal hemorheological properties in preeclampsia with repeated blood sampling from the diagnosis through the delivery until postpartum 72 hours among women diagnosed with early-onset preeclampsia compared to healthy uncomplicated pregnancies. We intended to identify one or more rheological parameters as potential factors giving additional information to the current diagnostic and screening method. Our secondary aims were to reveal the connection between maternal RBC properties and characteristics of pregnancy outcomes.

9. Materials and methods

13 non-smoking women diagnosed with early-onset preeclampsia based on the International Society for the Study of Hypertension in Pregnancy criteria and admitted to the Department of Obstetrics and Gynaecology, University of Pécs were involved in this prospective, case-control study. Control group was made up of 24 healthy, non-smoking, age- and gestation-matched women. In the first phase, we recruited 34 women suggested to be healthy and to have a pregnancy without complications intended for the control group. Finally, 10 women have been excluded due to peripartum complications (*Figure 8.*). In both groups, exclusion criteria were twin pregnancy, intrauterine developmental abnormality of the foetus, intrauterine infection, severe maternal anaemia, participation in another study, lack of signed informed consent. The study protocol was approved by the Regional and Local Research Ethics Committee at the Medical School, University of Pécs (6942-PTE 2018) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Anamnestic information, comorbid conditions, symptoms, gestational age at birth and laboratory parameters (electrolytes, markers of renal and hepatic function, inflammatory parameters, complete blood count) at the time of preeclampsia diagnosis and within 72 hours after the delivery were recorded. Furthermore, maternal physical data (height, bodyweight, body mass index, heart rate, systolic and diastolic blood pressure, mean arterial pressure) were also collected at enrolment. Neonatal physical parameters (birth weight, length, head

circumference, shoulder width) and Apgar score determined immediately after the birth (Apgar 1) and 5 minutes later (Apgar 5) were also documented.

In the patient group the first blood sample was drawn at diagnosis, in the control group at enrolment (26-34. weeks of gestation), later discussed as "initial" values. In both group blood samples were drawn two more times: within the 1st hour after delivery and 72±3 hours later. Every time 2x6 ml of peripheral blood from antecubital veins was collected into EDTA-Vacutainer tubes. The hemorheological measurements were performed within one hour after blood collection in the Hemorheological Laboratory of the University of Pécs under standardized conditions by the same investigator person. *Figure 8.* summarises the process of recruitment and data collection.

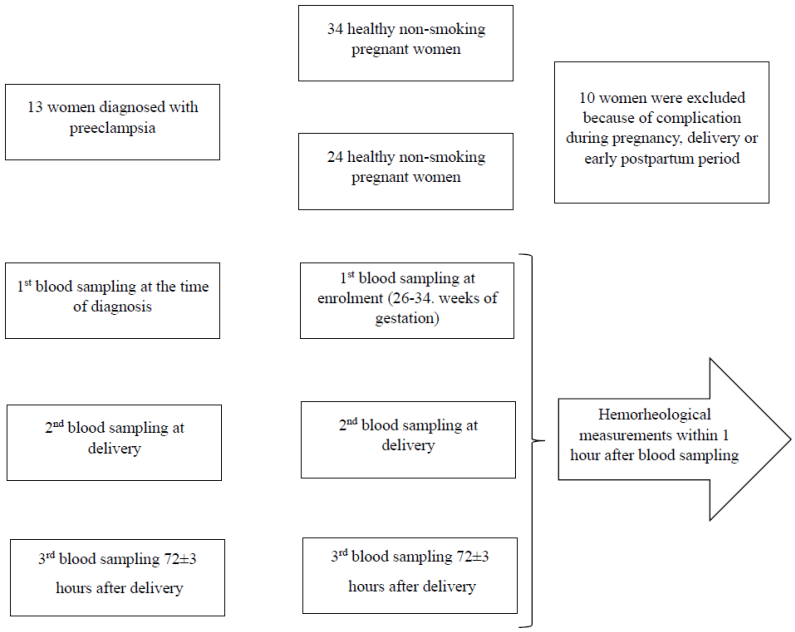

Figure 8. *Process of recruitment and data collection of women diagnosed with preeclampsia and control pregnant women.*

9.1. Hemorheological measurements

RBC aggregation was determined with two different methods. **Myrenne aggregometer** (model MA-1, Myrenne GmbH, Roetgen, Germany) applies and measures the infrared light transmission through the plasma gaps between the RBC aggregates on a transparent plate and a cone. The system rotates the injected 30 µl blood sample at high shear stress dispersing all pre-existing cell aggregates then instantly stops (M mode) or continues at reduced shear stress (M1 mode) to stimulate aggregation and measures the increasing light transmission proportional to the rate of RBC aggregate formation during stasis (*M index*) or at low shear (*M1 index*). The two dimensionless indices (M, M1) increase with enhanced erythrocyte aggregation. The **Laser-assisted Optical Rotational Cell Analyzer** (LORCA - R&R Mechatronics, Hoorn, Netherlands) determines the erythrocyte aggregation by detecting laser backscattering from the RBC aggregates. 1 ml blood sample is injected between the outer, rotating cylinder, and the inner, static cylinder followed by the RBC disaggregation at a high shear rate. The intensity of reflected light is measured for 120 seconds after the rapid stop of the motor. The aggregation index (AI), the aggregation half-time (t½ - which is the time

required to reach half of the maximum aggregation) and the threshold shear rate (γ - the smallest shear rate required for the complete disaggregation of RBCs) are calculated. LORCA is suitable to measure **erythrocyte deformability** on different shear stresses. 20 µl blood sample was diluted in a viscous medium and injected between the cylinders. A laser-diode is projected through the fluid, the light diffracts on the RBCs resulting in a diffraction pattern on a diaphragm. This is analysed by a video camera and a computer system. As a result of the applied increasing shear stress RBCs are elongated and the diffraction pattern is changing from circular to elliptical shape. Based on the measurements we could express RBC deformability as elongation index (EI) given at each shear stress.

9.2. Statistical analysis

Statistical analysis was evaluated by IBM SPSS Statistics® 27.0. Continuous variables are reported as mean ± standard deviation or medians with interquartile ranges. Categorical variables are reported as frequencies and percentages. The Kolmogorov-Smirnov test was applied to test for normality. Mann-Whitney U-test, Student T-test and Chi-square test were applied for data analysis. Bivariate correlation analysis was performed calculating Spearman's correlation coefficient (rho). The diagnostic power of the scores and parameters was assessed using AUC of the ROC curve. The predicted probabilities were calculated from the combination of initial AI and M variables produced by binary logistic regression analysis.

10. Results

The mean values of maximum measured systolic and diastolic blood pressure in the preeclampsia group were 180±18 mmHg and 112±13 mmHg. We observed significantly higher rate of preterm deliveries and infants with low birth weight, length, head circumference, and shoulder-width and more unfavourable Apgar 1 and 5 values in the preeclampsia group as expected (*Table 9. in the full thesis*).

We detected significantly elevated RBC count (4.29±0.12 vs. 3.91±0.05 G/L; p=0.009), and haemoglobin (126.58±2.60 vs. 117.91±1.38 g/l; p=0.003), and significantly lower mean corpuscular volume (MCV) (84.71±2.85 vs. 87.86±4.56 fl; p=0.037) at preeclampsia diagnosis compared to controls (*Figures 25-27. in the full thesis*).

RBC aggregation was significantly elevated at diagnosis (M: 9.8±0.4 vs. 8.5±0.2; p=0.007) and at delivery in preeclampsia (M: 10.7±0.8 vs. 8.0±0.4; p=0.002). In healthy pregnant women, the M index increased to 72 hours after birth compared to values at enrolment or delivery. This alteration was not observed among women with preeclampsia, where M values remained continuously high (*Figure 9. a.*).

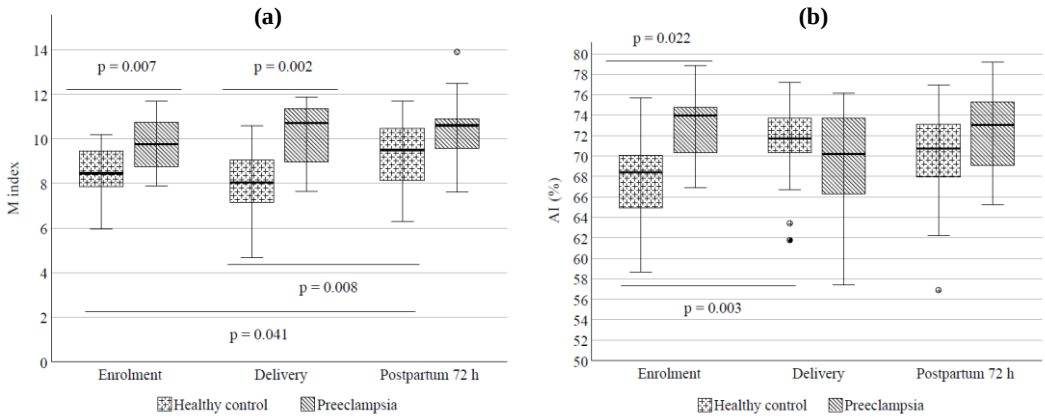

Figure 9 a-b. *RBC aggregation in preeclampsia and control group in the three investigated time point*

The RBC aggregation was more enhanced in preeclampsia at diagnosis compared to the control group (AI: 72.9±3.5% vs. 67.5±3.9%; p<0.001) (*Figure 9. b.*). Investigating the values in healthy pregnant women, significantly increased RBC aggregation was revealed from the first blood sampling at enrolment to the time of delivery (AI: 67.5±0.8% vs. 71.1±1.0% p=0.003), while this elevation was not visible in preeclampsia. Significant linear positive association was observed between initial AI measured at diagnosis and the gestational age of the neonate in the preeclampsia group (R^2=0.554; p=0.006) (*Figure 10.*)

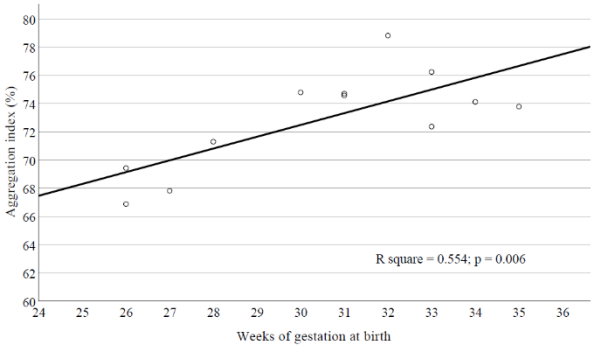

Figure 10. *Linear regression analysis of AI measured at preeclampsia diagnosis and weeks of gestation at birth*

Significantly reduced erythrocyte deformability was detected on medium shear stresses ($EI_{9.49Pa}$=0.554 vs. 0.559; $EI_{5.33Pa}$=0.496 vs. 0.504; EI_{3Pa}=0.421 vs. 0.430; p<0.05) at diagnosis of preeclampsia compared to the enrolment of healthy pregnant women. Analysing changes within groups we observed that the RBC deformability improved to 72 hours after delivery in preeclampsia compared to the values measured at diagnosis or during delivery, which kinetics was not ascertainable in the group of healthy pregnant women (*Table 10-11*. in the full thesis).

ROC analysis was carried out with maternal aggregation parameters in the first investigated time point to test the diagnostic power for preeclampsia. The analysis of initial AI indicated a cut-off point of 69.4% for preeclampsia with an AUC of 0.837 [0.684 – 0.990] (p=0.001) (sensitivity: 83.3%; specificity: 62.5%). ROC analysis of initial M values showed an AUC of 0.750 [0.576 – 0.924] (p=0.019) and indicated a cut-off as 8.39 (sensitivity: 90.9%; specificity: 50%) for preeclampsia. The predicted probabilities from combination of initial AI and M variables produced by binary logistic regression analysis showed slightly increased AUC

with 0.900 ([0.789 – 1.000] p<0.001) comparing the ones of AI or M value per se as classifiers for preeclampsia (*Figure 11.*).

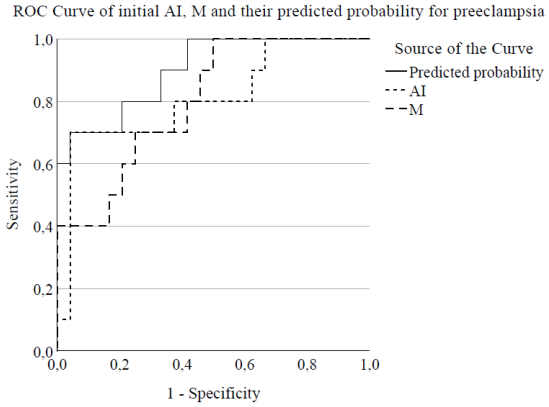

Figure 11. *ROC curve for preeclampsia diagnosis comparing initial AI and M values per se and their combination expressed as predicted probability*

11. Discussion

11.1. Summary of findings

Our research findings are intended to emphasise and confirm the role of maternal hemorheological alterations in pathophysiological processes affecting microcirculation in early-onset preeclampsia. Our investigations focused mainly on peripartum alterations of RBC properties including their aggregation and deformability. The most remarkable findings of our research are the potential diagnostic power of elevated AI and M index per se and in combination reflecting enhanced RBC aggregation with high sensitivity and acceptable specificity. We observed positive linear relationship between AI and gestational age at delivery in preeclampsia, which association was missing in healthy pregnancies. Significantly reduced initial EI values were observed on medium shear stresses reflecting impaired erythrocyte deformability at diagnosis of preeclampsia. RBC deformability improved within three days after delivery in women with preeclampsia compared to the values measured at diagnosis or during delivery.

11.2. Routinely measured RBC laboratory parameters

During normal pregnancy, total blood volume, plasma volume, and RBC mass are extensively increasing. Additionally, the plasma volume is elevating proportionally more than the RBC mass, resulting in lower haemoglobin concentrations from physiological haemodilution. We observed slightly elevated RBC count and haemoglobin values in early-onset preeclampsia, which results are in line with previous findings. Initial MCV was slightly decreased in our preeclampsia group compared to controls, contrary to previous investigations, where significantly increased MCV were reported in preeclampsia. Importantly, in these studies, the mean gestational age was higher in both groups than in our study. According to a recent publication analysing the osmotic and mechanical stability of erythrocytes, it is suggested that erythrocytes with lower volume and lower haemoglobin content are osmotically more stable. They assumed that lower MCV values could be a mechanism of compensatory

mechanical selection, which is beneficial in preeclampsia. However, the desired range of MCV throughout pregnancies complicated by preeclampsia remains unclear.

11.3. Erythrocyte aggregation

According to our results, elevated initial maternal AI and M values were proved to be the most promising indicators of early-onset preeclampsia with high sensitivity and acceptable specificity. Moreover, we gained more favourable AUC by their combination compared to their individual analysis. The elevated RBC aggregation at preeclampsia diagnosis compared to controls refer to impaired microcirculation in early-onset preeclampsia, which is also supported by the relationship between AI and gestational age at birth. This association suggests that the longer the pathologic pregnancy persists, the worse the AI values are, reflecting enhanced maternal RBC aggregation. We did not observe significant relationship in healthy pregnancies concerning their AI value and their gestational age at enrolment, contrary to preeclampsia. These observations suggest that in normal pregnancy, the gestational age alone did not influence AI values, this association was specific to preeclampsia. It should be noted that initial AI values in both groups were independent of haemoglobin values, so they did not affect our measurement results in either group. Analysing the changes within the groups, we can conclude that RBC aggregation increased within the first 72 post-partum hours in healthy pregnancies. Contrary, continuously elevated RBC aggregation was observed in preeclampsia, and we did not find a significant difference between the three investigated time points. Elevated RBC aggregation can be attributed to plasma protein levels and in preeclampsia mainly to intrinsic alterations of RBC cell membrane such as reduced sialic acid content that weakens repulsive forces and conformational changes of the membrane enhancing erythrocyte aggregation.

11.4. Erythrocyte deformability

Initial EI values at medium shear stresses were decreased in preeclampsia reflecting altered RBC deformability. The relatively rapid improvement of deformability in the postpartum period in preeclampsia may presumably occur due to the termination of gravidity, which has maintained the disease. It can therefore be hypothesised that RBC deformability may be a sensitive and early marker of the normalisation of maternal microcirculation after delivery. The reason for reduced RBC deformability in preeclampsia is suggested to be chronic inflammation and hypoxia, which leads to increased concentration of free radicals inducing changes in RBC membrane properties and subsequent increase of intracellular Ca^{2+}. In addition, an increased Ca^{2+} pump activity leads to adenosine triphosphate depletion in RBCs resulting in poor deformability.

11.5. Previous literature

Previous studies on RBC deformability and aggregation in preeclampsia seem to be in conflict. Some authors reported decreased erythrocyte deformability and uteroplacental hypoperfusion with subsequent RBC membrane damage in preeclampsia. Others did not observe any significant deformability alteration. Increased erythrocyte aggregation was also described in preeclampsia, while other authors observed no significant deterioration. In line with our results, L. Heilmann et al. found enhanced RBC aggregation and reduced deformability

in severe preeclampsia suggesting that hemorheological parameters play an important role in the microcirculation of the intervillous space of the placenta.

Most of the studies dealing with hemorheological alterations in preeclampsia were published more than 2-3 decades ago. As preeclampsia definitions, the measurement methods and interpretation of results may have changed since then, it is necessary to re-evaluate these results. Moreover, the diagnostic criteria for early-onset preeclampsia have been broadened and clarified. The distinction between the early- and late-onset forms is increasingly recognised since they have a different pathophysiological background and clinical features, thus they should be investigated completely separately. Early-onset preeclampsia is suggested to be originated mainly from defective placentation, whilst late-onset form may develop due to maternal genetic predisposition to cardiovascular and metabolic disease. Therefore, it would be desirable to treat the two types separately from a hemorheological point of view as well.

Regarding RBC aggregation, previous investigations applied mostly Myrenne aggregometer per se, while only a few reported results were measured by LORCA. To the best of our knowledge, there are no data in the literature that examine these two measurement methods simultaneously in early-onset preeclampsia as we have performed. It can be seen from our report that the two types of measurement methods gave similar results in terms of RBC aggregation, moreover, their combination would even raise the diagnostic power.

11.6. Screening

In the past decade, extensive efforts have been made to develop an efficient screening algorithm to identify high-risk patients who would benefit from acetylsalicylic acid prophylaxis. Currently, the best screening model in the first trimester combines multiple factors and specific biomarkers, whose determination is effortful, time-consuming, and requires costly measurements and contribution of qualified professionals. In contrast, we investigated a cheap method with easy implementation and indicators that are quantitative, objective, and can be blinded to other clinical characteristics. Excluding the purchase of the instruments, hemorheological tests costs are minimal and can be easily performed. The above-detailed measurements require a small amount of blood sample and provide quickly obtainable results. Therefore, further investigations are suggested to reveal whether RBC aggregation and deformability parameters, especially AI and M values possess the potential for susceptibility or risk biomarkers in the early stage of preeclampsia before the onset of symptoms. Moreover, their inclusion in the screening algorithm should be considered.

11.7. Strengths, limitations, and future perspectives

The strength of our study is the prospective, case-control design with repeated blood sampling in the peripartum period to evaluate the kinetics and changes of RBC properties. Using two methods together (Myrenne and LORCA) provides more reliable information on RBC aggregation properties. The low total number of enrolled patients and its single-centre nature with local treatment strategies and guidelines could limit the generalisability of our findings. RBC properties could help in the prognostication of early-onset preeclampsia, but further investigations are warranted to confirm the prognostic role before the onset of symptoms. Long-term follow-up involving higher number of patients is suggested to investigate

the potential role of peripartum hemorheological alterations in the development of cardiovascular complications in later life of the mother. Examination of umbilical cord blood and neonatal blood samples may contribute to the prediction of foetal complications.

12. Summary of novel findings

12.1. Novel and conventional biomarkers for post-resuscitation prognosis

- Initial ADMA levels independently predict early death within 3 days after resuscitated cardiac arrest.
- Elevated ADMA levels are associated with persistent vegetative state or brain death during post-resuscitation care.
- Significant positive correlation was revealed between initial ADMA and SAPS II score.
- CK-18, CCCK-18 and NSE are not associated with survival or neurological outcome after cardiac arrest in the cohort of unselected resuscitated patients.
- CK-18 levels were persistently elevated with decreased CCCK-18/CK-18 ratio on the first three days referring to a large extent of cell death dominantly due to necrosis.
- 72-hour CK-18 level showed significant correlation with SAPS II and SOFA scores.
- Initial lactate level predicts 30-day mortality and poor neurological outcome and provides similarly useful information per se as SAPS II and SOFA scores.

12.2. Maternal hemorheological properties in early-onset preeclampsia

- Elevated AI and M index reflecting increased erythrocyte aggregation were proved to be the most promising indicators in maternal blood samples with high sensitivity for early-onset preeclampsia diagnosis.
- Significant positive linear relationship was found between AI and gestational age at birth in preeclampsia, which association was missing in healthy pregnancies.
- Lower EI values were observed at preeclampsia diagnosis compared to normal pregnancy reflecting deteriorated RBC deformability, which improved rapidly in postpartum period in preeclampsia group.

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14. Scientometrics

Scientific papers:

- Total: 9
- English language papers: 7

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List of publications:

First author papers upon which this thesis relies:

1. **Csiszar, Beata**; Marton, Zsolt; Riba, Janos; Csecsei, Peter; Nagy, Lajos; Toth, Kalman; Halmosi, Robert; Sandor, Barbara; Kenyeres, Peter (corresponding author); Molnar, Tihamer. L-Arginine, asymmetric and symmetric dimethylarginine for early outcome prediction in unselected cardiac arrest victims: a prospective cohort study. INTERNAL AND EMERGENCY MEDICINE (2021). Published: 03 June 2021; https://doi.org/10.1007/s11739-021-02767-z **Q1;** IF: 3.397 (2020) H-index: 47
2. **Csiszár, Beáta**; Németh, Álmos Márton, Zsolt; Riba, János; Csécsei, Péter; Molnár, Tihamér; Deres, László; Halmosi, Róbert; Tóth, Kálmán; Kenyeres, Péter. A citokeratin-18 sejthalálmarker vizsgálata sikeres cardiopulmonalis resuscitatión átesett betegpopulációban. ORVOSI HETILAP 161: 1 pp. 26-32. , 7 p. (2020); **Q4**; IF: 0.540 (2020) H-index: 21

Other papers:

1. Meggyes, Matyas; Nagy, David U.; Szigeti, Brigitta; **Csiszar, Beata**; Sandor, Barbara; Tamas, Peter; Szereday, Laszlo. Investigation of mucosal-associated invariant T (MAIT) cells expressing immune checkpoint receptors (TIGIT and CD226) in early-onset preeclampsia. EUROPEAN JOURNAL OF OBSTETRICS GYNECOLOGY AND REPRODUCTIVE BIOLOGY 252 pp. 373-381., 9 p. (2020)
2. Szakács, Zsolt; **Csiszár, Beáta**; Nagy, Mátyás; Farkas, Nelli; Kenyeres, Péter; Erős, Adrienn; Hussain, Alizadeh; Márta, Katalin; Szentesi, Andrea; Tőkés-Füzesi, Margit et al. Diet-dependent and diet-independent hemorheological alterations in celiac disease: A case-control study CLINICAL AND TRANSLATIONAL GASTROENTEROLOGY 11: 11 Paper: e00256, 11 p. (2020)
3. Meggyes, Matyas; Miko, Eva; Lajko, Adrienn; **Csiszar, Beata**; Sandor, Barbara; Matrai, Peter; Tamas, Peter; Szereday, Laszlo. Involvement of the PD-1/PD-L1 Co-Inhibitory Pathway in the Pathogenesis of the Inflammatory Stage of Early-Onset Preeclampsia. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES 20 : 3 Paper: 583 , 11 p. (2019)
4. Szakács, Zsolt; **Csiszár, Beáta**; Kenyeres, Péter; Sarlós, Patrícia; Erőss, Bálint; Hussain, Alizadeh; Nagy, Ágnes; Kőszegi, Balázs; Veczák, Ibolya; Farkas, Nelli et al. Haemorheological and haemostatic alterations in coeliac disease and inflammatory bowel disease in comparison with non-coeliac, non-IBD subjects (HERMES): a case-control study protocol. BMJ OPEN 9: 3 Paper: e026315, 8 p. (2019)
5. Biro, K; Sandor, B; Kovacs, D; **Csiszar, B**; Vekasi, J; Totsimon, K; Toth, A; Koltai, K; Endrei, D; Toth, K et al. Lower limb ischemia and microrheological alterations in patients with diabetic retinopathy. CLINICAL HEMORHEOLOGY AND MICROCIRCULATION 69: 1-2 pp. 23-35., 13 p. (2018)
6. Kovacs, David; **Csiszar, Beata**; Biro, Katalin; Koltai, Katalin; Endrei, Dora; Juricskay, Istvan; Sandor, Barbara; Praksch, Dora; Toth, Kalman; Kesmarky, Gabor. Toe-brachial index and exercise test can improve the exploration of peripheral artery disease. ATHEROSCLEROSIS 269 pp. 151-158., 8 p. (2018)
7. Koltai, K; Biró, K; Kovács, D; **Csiszár, B**; Tóth, K; Késmárky, G. A cilostazol hatásmechanizmusa és szerepe a perifériás verőérbetegség kezelésében. LEGE ARTIS MEDICINAE 25: 4-5 pp. 177-181., 5 p. (2015)

Conference abstracts related to the topic of the thesis:

1. **Beata Csiszar,** Gergely Galos, Peter Kenyeres, Kalman Toth, Barbara Sandor. Maternal hemorheological changes in early-onset preeclampsia. 2nd Joint Meeting of ESCHM-ISCH-ISB 2021, Fukuoka, Japan, online conference, July 4-7, 2021
2. **Beata Csiszar**, Gergely Galos, Simone Funke, Miklos Koppan, Matyas Meggyes, Laszlo Szereday, Peter Kenyeres, Kalman Toth, Barbara Sandor. Maternal hemorheological changes in early-onset preeclampsia. Magyar Haemorheologiai Társaság XXVII. Kongresszusa, online, 2021. április 23.
3. **Csiszár Beáta**, Márton Zsolt, Riba János, Csécsei Péter, Molnár Tihamér, Deres László, Kőszegi Tamás, Tóth Kálmán, Halmosi Róbert, Kenyeres Péter. A cytokeratin-18 prognosztikus szerepének vizsgálata posztreszuszcitációs ellátás során. Magyar Kardiológusok Társasága 2020. évi Tudományos Kongresszusa online. 2020. november 11-14.
4. **Csiszar Beata**, Nemeth Almos, Marton Zsolt, Riba Janos, Csecsei Peter, Molnar Tihamer, Deres Laszlo, Toth Kalman, Kenyeres Peter. Prognostic value of systemic cell death biomarkers after successful cardiopulmonary resuscitation. RESUSCITATION 142: Suppl. 1 pp. e96-e97. Paper: AP162 (2019)
5. **Csiszár Beáta**, Németh Álmos, Márton Zsolt, Riba János, Csécsei Péter, Molnár Tihamér, Deres László, Tóth Kálmán, Kenyeres Péter. A cytokeratin-18 szerepének vizsgálata sikeres cardiopulmonalis resuscitation átesett betegpopulációban. Magyar Aneszteziológiai és Intenzív Terápiás Társaság Nemzeti Kongresszusa MAITT továbbképző napok, Anesztexpo 2019. Hotel Azúr, Siófok 2019. május 23–25.
6. **Csiszár Beáta**, Kevey Dóra Kinga, Császár András, Vida Gabriella, Funke Simone, Szereday László, Meggyes Mátyás, Tóth Kálmán1, Sándor Barbara. Preeclampsiás édesanyák és újszülöttjeik hemoreológiai paramétereinek vizsgálata. Magyar Haemorheologiai Társaság XXVI. Kongresszusa. 2019. április 12-13. Pécs, Hotel Therapia.
7. **Csiszár Beáta**, Németh Álmos, Márton Zsolt, Csécsei Péter, Molnár Tihamér, Tóth Kálmán, Kenyeres Péter. Apoptózis és nekrózis markerek vizsgálata sikeres cardiopulmonalis resuscitation átesett

betegpopulációban. A Magyar Belgyógyász Társaság Dunántúli Szekciójának LX. Vándorgyűlése és XX. Szekszárdi Kardiológiai Nap, 2019. március 28-30.

Other conference abstracts:

1. **Beata Csiszar**, Kinga Totsimon, Peter Kenyeres, Kalman Toth, Zsolt Marton. Hemorheological parameters and mortality in critically ill patients. Joint Conference of Three Societies: The European Society for Clinical Hemorheology and Microcirculation, The International Society of Clinical Hemorheology and The International Society of Biorheology (ESCHM-ISCH-ISB-2018) July 2-6, 2018 in Krakow, Poland.
2. **Csiszár, B**; Németh, Á; Riba, J; Márton, Zs; Tóth, K; Kenyeres, P: Miből lesz a tamponád – kezelés vagy kezeletlen betegség szövődménye? CARDIOLOGIA HUNGARICA 48: Suppl. C pp. C 57-C 57., 1 p. (2018)
3. Késmárky, G; Kovács, D; **Csiszár, B**; Bíró, K; Koltai, K; Endrei, D; Tóth, K. Non-invazív módszerek és terheléses vizsgálatok szerepe az alsó végtagi panaszok differenciál diagnosztikájában. ÉRBETEGSÉGEK/HUNGARIAN JOURNAL OF VASCULAR DISEASES 24: 2 pp. 12-12., 1 p. (2017)
4. Kovács, D; **Csiszár, B**; Juricskay, I; Bíró, K; Koltai, K; Endrei, D; Praksch, D; Tóth, K; Késmárky, G: The role of exercise tests in the evaluation of vascular patients for lower limb ischemia. CARDIOLOGIA HUNGARICA 47: Suppl. p. n/a (2017)
5. Kovács, D; **Csiszár, B**; Juricskay, I; Bíró, K; Koltai, K; Endrei, D; Praksch, D; Tóth, K; Késmárky, G: Non-invazív módszerek és terheléses vizsgálatok szerepe perifériás ütőérbetegek végtag iszkémiájának diagnosztikájában. ÉRBETEGSÉGEK/HUNGARIAN JOURNAL OF VASCULAR DISEASES 24: 2 pp. 11-11., 1 p. (2017)
6. Kovács, D; **Csiszár, B**; Juricskay, I; Bíró, K; Koltai, K; Endrei, D; Praksch, D; Tóth, K; Késmárky, G. Terheléses vizsgálatok szerepe perifériás űtőérbetegek végtag-iszkémiájának diagnosztikájában. CARDIOLOGIA HUNGARICA 47 p. C129 (2017)
7. Biró, K; Kovács, D; **Csiszár, B**; Tótsimon, K; Sándor, B; Tóth, A; Koltai, K; Vékási, J; Tóth, K; Késmárky, G. Klaudikáló és nem klaudikáló diabéteszes betegek alsó végtagi keringésének vizsgálata. CARDIOLOGIA HUNGARICA 46: Suppl. F Paper: F89 (2016)
8. Késmárky, G; Kovács, D; **Csiszár, B**; Biró, K; Koltai, K; Endrei, D; Battyáni, I; Menyhei, G; Tóth, K. A noninvaziv angiológiai vizsgálatok szerepe a döntéshozatalban perifériás ütőérbetegnél: esetismertetés. CARDIOLOGIA HUNGARICA 46: Suppl. F Paper: F67 (2016)
9. Kesmarky, G; Biro, K; Koltai, K; Kovacs, D; **Csiszar, B**; Kovacs, M; Totsimon, K; Sandor, B; Toth, A; Toth, K. Haemorheological and circulatory investigations in peripheral artery diseases. ÉRBETEGSÉGEK/HUNGARIAN JOURNAL OF VASCULAR DISEASES 22: Suppl. 1 pp. 52-52., 1 p. (2015)
10. Késmárky, G; Bíró, K; Koltai, K; Kovács, D; **Csiszár, B**; Endrei, D; Tóth, K. Infúziós kezelés perifériás ütőérbetegségekben. ÉRBETEGSÉGEK / HUNGARIAN JOURNAL OF VASCULAR DISEASES 22: 2 pp. 53-53., 1 p. (2015)
11. Koltai, K; Biró, K; Kovács, D; **Csiszár, B**; Tóth, K; Késmárky, G. A transcutan parciális szöveti oxigéntenzió mérés és a lézer-doppler-áramlásmérés szerepe diabeteses betegekben (8+2'). MAGYAR BELORVOSI ARCHIVUM 68: Suppl. 1 pp. 18-18., 1 p. (2015)
12. Kovacs, D; Biro, K; **Csiszar, B**; Totsimon, K; Sandor, B; Toth, A; Koltai, K; Vekasi, J; Toth, K; Kesmarky, G. Examination of lower limb tissue perfusion in diabetic patients with retinopathy. ÉRBETEGSÉGEK / HUNGARIAN JOURNAL OF VASCULAR DISEASES 22: Suppl. 1 pp. 35-35., 1 p. (2015)

Book, editorial:

1. **Csiszár, Beáta;** Hankó, Csilla; Kajos, Luca Fanni; Mező, Emerencia**:** Medical Conference for PhD Students and Experts of Clinical Sciences 2021: Book of Abstracts. Pécs, Hungary: Doctoral Student Association of the University of Pécs (2021), 124 p. ISBN: 9789634296539
2. **Csiszár, B;** Hankó, Cs; Kajos, L F; Kovács, O B; Mező, E; Szabó, R; Szabó-Guth, K: IX. Interdiszciplináris Doktorandusz Konferencia 2020 Absztraktkötet: 9th Interdisciplinary Doctoral

Conference 2020 Book of Abstracts. Pécs, Hungary: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2020), 384 p. ISBN: 9789634295822

3. **Csiszár, Beáta**; Hankó, Csilla; Kajos, Luca Fanni; Mező, Emerencia: IX. Interdiszciplináris Doktorandusz Konferencia 2020 Tanulmánykötet; 9th Interdisciplinary Doctoral Conference 2020 Conference Book. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2020), 595 p. ISBN: 9789634295839
4. **Csiszár, Beáta**; Hankó, Csilla; Kajos, Luca Fanni; Mező, Emerencia: Medical Conference for PhD Students and Experts of Clinical Sciences: Book of Abstracts. Pécs, Hungary: Doctoral Student Association of the University of Pécs (2020), 83 p. ISBN: 9789634295440
5. Bódog, Ferenc; **Csiszár, Beáta**: VIII. Interdiszciplináris Doktorandusz Konferencia 2019. Absztraktkötet; 8th Interdisciplinary Doctoral Conference 2019. Book of Abstracts. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2019), 185 p. ISBN: 9789634293743
6. **Csiszár, Beáta**; Bódog, Ferenc: Medical Conference for PhD Students and Experts of Clinical Sciences: Book of abstracts. Pécs, Hungary: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2019), 87 p. ISBN: 9789634292913
7. **Csiszár, Beáta**; Bódog, Ferenc; Mező, Emerencia; Závodi, Bence: VIII. Interdiszciplináris Doktorandusz Konferencia 2019. Tanulmánykötet; 8th Interdisciplinary Doctoral Conference 2019. Conference Book. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2019) , 327 p. ISBN: 9789634293729
8. Bódog, Ferenc; **Csiszár, Beáta**; Hayden, Zsófa; Kovács, Olivér; Rácz, Tamás. VII. Interdiszciplináris Doktorandusz Konferencia 2018. Tanulmánykötet; 7th Interdisciplinary Doctoral Conference 2018. Conference Book. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2018), 492 p. ISBN: 9789634292111
9. Bódog, Ferenc; **Csiszár, Beáta**; Pónusz, Róbert: Medical Conference for PhD Students and Experts of Clinical Sciences Book of Abstracts. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2018) ISBN: 9789634292760
10. Bódog, Ferenc; **Csiszár, Beáta**; Hayden, Zsófia; Mészáros, Orsolya; Sapolov, Anatolij; Pónusz, Róbert. VII. Interdiszciplináris Doktorandusz Konferencia 2018 Absztraktötet: Digitális, bővített kiadás; 7th Interdisciplinary Doctoral Conference 2018 Book of Abstracts: Digital, extended edition. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2018), 207 p. ISBN: 9789634292098
11. Bódog, Ferenc; **Csiszár, Beáta**; Hegyi, Dávid; Pónusz, Róbert. DKK17-Doktoranduszok a Klinikai Kutatásokban Absztraktkötet. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2017), 89 p. ISBN: 9789634291602