Prognostic value of cell-free DNA in plasma of out-of-hospital cardiac arrest survivors at ICU admission and 24 hours post-admission

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ABSTRACT

Cell-free DNA has been associated with outcome in several acute conditions including two reports concerning the outcomes after cardiac arrest that found association of circulating DNA quantities at admission with mortality. The origins of cell-free DNA are primarily necrosis and apoptosis, which in cardiac arrest occur during ischemia ("no-flow" and "low-flow" period), during reperfusion injury and as a consequence of post-arrest inflammatory response. Respecting the facts that significant cellular damage may occur during the post-arrest period, and that that damage might be reduced by mild therapeutic hypothermia, we investigated the prognostic value of cell free DNA at ICU admission and 24 hours after admission.

A prospective study was conducted in three university associated intensive care units and included patients resuscitated from non-traumatic out-of-hospital cardiac arrest. Patient data were collected in accordance with the Utstein protocol. Therapeutic hypothermia was performed according to ICU policies.
Blood for cell-free DNA quantification was sampled at admission and at 24±1 hour after admission. Outcome measures were hospital morality and cerebral performance expressed with CPC scale at discharge.

Inclusion criteria were met in 67 patients; 24-hour mortality was 37.3% and hospital mortality 71.6%. The following variables were associated with 24-hour mortality in univariate analysis: asystole as the presenting rhythm, "no-flow" time, "low-flow" time and cell-free DNA at admission (median 0.081 in survivors vs. 0.160 ng/µl in non-survivors; P=0.038). Multivariate analysis that included the above variables showed that no-flow time and low-flow time were independently associated with 24-hour mortality. Hospital mortality was associated with the following factors: "low flow" time, coronary intervention, cell-free DNA at ICU admission and at 24 hours after admission (0.042 vs. 0.188 ng/µl; P=0.048). ROC curve for cell-free DNA 24 h post-admission showed sensitivity of 81.0% and specificity of 78.3% for the cut-off value of 0.115 ng/µl. Multivariate analysis showed that "low-flow" time and cell-free DNA at 24 hours after ICU admission were independently associated with hospital mortality. Cell free DNA showed different dynamics in patients who were and who were not treated with mild therapeutic hypothermia: it decreased in treated patients and slightly increased in non-treated patients.

Cell-free DNA quantity at ICU admission and 24 h after admission is associated with hospital mortality. Further studies will need to additionally investigate possible practical use of this new laboratory marker in patients resuscitated from cardiac arrest.

INTRODUCTION

Successful resuscitation from cardiac arrest is associated with high morbidity and mortality [1]. Some biochemical markers, such as neuron-specific enolase [2] and protein S-100B [3], have been associated with outcome after cardiac arrest, but predicting outcome in such patients remains an important issue, medical, ethical and economical. Over pessimistic predictions may deny active support to patients with a chance for recovery and too optimistic predictions could burden the patients and the intensive care units with futile intensive care.

Cell-free plasma DNA has been investigated as a diagnostic tool in various conditions [4], mainly chronic: autoimmune diseases [5] and cancer [6, 7], but it was also investigated and associated with outcome in several acute conditions such as trauma [8], stroke [9], myocardial infarction [10], sepsis [11] and acute pancreatitis [12, 13].

Cell death by apoptosis or necrosis is supposed to be the main source of circulating DNA [6, 7, 14], although all the aspects of its origins and clearance are not fully understood. Cardiac arrest and post-resuscitation period is associated with cellular necrosis due to hypoxia during the no-flow period, but substantial cellular damage also occurs after circulation has been restored [1, 15, 16], as a consequence of reperfusion injury and inflammatory response. Harmful processes that occur during the post-resuscitation period have in the past years been targeted with mild therapeutic hypothermia (MTH) which was proven to reduce mortality [17] and was adopted as a recommended treatment in such patients [18].

Two recent papers [19, 20] reported that cell-free DNA, quantified early after resuscitation from cardiac arrest, is associated with mortality. In the later paper [19], DNA was also quantified 24 hours and 72 hours after arrest, but no significant difference associated with hospital mortality was found. Respecting the facts that significant cellular damage may occur during the post-arrest period [1], and that that damage might be reduced by MTH [18], we investigated the prognostic value of cell free DNA at ICU admission and 24 hours after admission.

PATIENTS AND METHODS

This was a prospective study conducted in three intensive care units in hospitals affiliated to the University of Zagreb School of Medicine. We included consecutive patients who were admitted after a non-traumatic out-of-hospital cardiac arrest and successful resuscitation. Inclusion period started on Feb 1st 2010 and ended on Jan 31st 2012. The inclusion criteria were: 1) age $\geq$ 18 years; 2) successful recovery of spontaneous circulation within 60 minutes from collapse. The exclusion criteria were: 1) end-stage chronic disease or “don-not-resuscitate” order; 2) survival for less than 12 hours after the arrest; 3) myocardial infarction (MI), stroke or arrest within 30 days before the index event, but not if MI was the admission diagnosis of the index hospitalization 4) failure to obtain informed consent from the family.

Patient data were collected in accordance with the Utstein protocol [21]. Cardiac arrest was defined as absence of spontaneous respiration and pulse with one of the following rhythms: ventricular fibrillation (VF), ventricular tachycardia (VT), pulseless electrical activity (PEA) and asystole.
Therapeutic hypothermia was performed at the discretion of admitting physicians and according to ICU policies. If implemented, it was maintained at target core temperature 33±1 ºC for 24 hours; protocols and methods for cooling and re-warming depended on participating ICUs' policies, but all units used cold saline infusions and external cooling.

Blood samples for cell-free DNA quantification were drawn 1) during the first 30 min from ICU admission and 2) at 24±1 hour after admission. Plasma was separated by centrifugation immediately after blood draw and stored until analysis at -80 ºC.

Outcome measures were hospital morality and neurological outcome expressed with cerebral performance category (CPC) scale [22] at hospital discharge. Six-month mortality was also assessed.

Free DNA assay
DNA was extracted from 100 µl of plasma using DNeasy® Blood and Tissue Kit (QIAGEN GmbH, Germany) according to the manufacturer's protocol.
Real time PCR was performed using Quantifiler™ Human DNA Quantification kit (Applied Biosystems, USA). A coding region of the human telomerase reverse transcriptase gene (hTERT locus located on chromosome 5) was used to quantify total DNA. Detailed PCR protocol is described in our previous work [12].
Amplification data were collected and analyzed with an ABI Prism 7000 Sequence Detection System (SDS) instrument (Applied Biosystems, USA). The cycle threshold value (Ct) was measured in all cases. Each sample was analyzed in duplicate, and multiple negative reaction blanks were included in every analysis for both sample extraction and amplification stages. Calibration curve was analyzed on the same reaction plate for each run using quantification standard dilutions [12].

Statistical analyses
All analyses were performed using SPSS v17.0 software (SPSS, Chicago, Ill., USA).
Due to small sample size, continuous data are presented as median with interquartile range, discrete variables as absolute and relative frequencies.
Wilcoxon's test was used for group comparisons of continuous variables; chi-squared test for categorical variables. For correlation analyses, Spearman's rank correlation was used.
Multivariate analyses – multiple logistic regression – was performed after univariate analyses to investigate which variables were independently associated with an outcome. The analyses included variables associated with the dependent variable in univariate analysis that did not have significant correlation between them. Statistical significance was set at $\alpha = 0.05$.

The study was approved by ethics committees of the participating hospitals. Patients' next of kin was asked for consent for inclusion in the study; patients who recovered consciousness were asked to confirm consent for participation in the study.

RESULTS

There were 116 resuscitated patients admitted to participating ICUs during the inclusion period. We excluded: 14 terminally ill, 4 with recent MI or stroke, 12 for whom we could not get consent and 19 patients who died within 12 hours after arrest.

The remaining 67 patients had 24-hour mortality, hospital mortality and six-month mortality of 37.3%, 71.6% and 72.9% respectively. There were no differences in mortalities between the participating centres. The demographic data, comorbidities, arrest data, treatment and outcome data are depicted in Table 1.

Univariate analysis showed that the following variables were associated with 24-hour mortality: initial rhythm (asystole in 76% survivors, 36% non-survivors; P=0.006), defibrillation performed (24% survivors, 52.3% non-survivors; P=0.042), "no-flow" time (median 4 min for survivors vs. 5 min for non-survivors; P=0.008), "low-flow" time (median 22 min for survivors vs. 31 min for non-survivors; P<0.001) and cell-free DNA at ICU admission (median 0.081 ng/μL in survivors vs. 0.160 ng/μL in non-survivors; P=0.038).

Inter-variable analyses showed that initial rhythm was associated with low-flow time and with performance of defibrillation; the two variables were thus excluded from the multivariate model that showed that no-flow time and initial rhythm were independently associated with 24-hour mortality (Table 2).

Hospital mortality was associated with following factors: "low flow" time, coronary intervention, cell-free DNA at ICU admission and at 24 hours after admission (Table 1).

Receiver operating characteristic (ROC) curves were constructed to evaluate predictive value for hospital mortality (Figure 1). Cell free DNA at 24 hours after admission had sensitivity of 81.0% and specificity of 78.3% for the optimal cut-off value of 0.115 ng/μL; area under ROC curve was 0.762 (95% confidence interval 0.61-0.88). Cell-free DNA quantified at admission had the optimal cut-off at 0.092 with sensitivity of 70% and specificity of 60.4%; area under ROC curve was 0.636 (95% CI 0.511-0.750).

Multivariate analysis that included variables associated with hospital mortality showed that "Low-flow" time and cell-free DNA at 24 hours after ICU admission (dichotomised according to the cut-off value of 0.115 ng/μL, optimal from ROC curve) were independently associated with hospital mortality (Table 3).

Cell-free DNA at 24 hours was lower in patients who were treated with mild therapeutic hypothermia (median 0.038 ng/μL, IQR 0.0173-0.084) compared to patients who were not (median 0.172 ng/μL, IQR 0.113-0.423; P=0.048).

As shown in Figure 2, the quantity of cell-free DNA had a tendency to rise from admission (median 0.081 ng/μL, IQR 0.038-0.185) to 24 hours after admission (median 0.115 ng/μL, IQR 0.048-0.309; P=0.088). In the subgroup of patients who were not treated with MTH, the increase in DNA quantity was significant (median 0.086 ng/μL, IQR 0.057-0.222 vs. median. 0.167 ng/μL, IQR 0.113-0.423; P=0.034). Patients who were treated with MTH had a trend for lower DNA quantities at 24 hours than at

admission (median 0.038 ng/μL; IQR 0.029-0.143 vs. 0.053 ng/μL, IQR 0.017-0.084; P=0.079).
The difference in DNA quantity from admission to 24 hours after admission was negative in patients who were treated with MTH (median -0.041) and positive in patients who were not (median 0.87 ng/μL, P=0.039)

Cerebral performance categories had significant positive correlation with plasma cell-free DNA quantified 24-hours after admission (r=0.351, P=0.020) and no correlation with plasma cell-free DNA quantified at admission (P=0.587).

At six months after the index event, survivors also had significantly lower cell-free DNA levels quantified at ICU admission (median 0.302 ng/μL vs. 0.335 ng/μL: P=0.010) and at 24 hours after admission (median 0.248 ng/μL vs. 0.368 ng/μL; P=0.048).

DISCUSSION

The presented study shows that plasma cell-free DNA at ICU admission is associated with 24-hour, hospital mortality and six-month mortality. Cell-free DNA quantified at 24 hours after admission was associated with hospital mortality and CPC score at discharge. We also found that treatment with mild therapeutic hypothermia is associated with different cell-free DNA temporal pattern i.e. DNA quantities rose in patients who had not been treated with MTH.

Only patients with out-of hospital cardiac arrest were included, and a relatively high mortality of almost 70% was observed. The reasons for high mortality are speculative. Low ratio (only about 20%) of patients with VF as initial rhythm probably is a part of the answer since those patients have better prognosis. Relatively long response times by our emergency teams could also be in part responsible to high mortality, but also to low ratio of VF. Other factors, such as (non-) implementation of MTH also may contribute.

The dynamics of cell-free DNA in plasma after cardiac arrest are not clearly understood. Circulating DNA originates from cell necrosis due to hypoxia during the arrest, but also from necrosis and apoptosis of cells after the arrest, consequent to complex mechanisms that include inflammation, free radical formation, altered calcium homeostasis, excitotoxicity, pathological protease cascades and activation of cell death signalling [1].

The anoxic and post-anoxic injuries occur in all organs and cell-free DNA originates from all tissues, unlike some other conditions in which circulating DNA was associated with outcome, such as stroke [9], myocardial infarction [10] and acute pancreatitis [13]. In the post-arrest patients, the cerebral and myocardial damage determine the patients' outcome and although circulating DNA originates from all tissues, its quantity is still in association with mortality.

Mild therapeutic hypothermia aims to reduce post-arrest damage and consequently improve the outcome. Different dynamics of circulating DNA quantities in patients treated with MTH should reflect the effects of hypothermia on the detrimental processes in the post-arrest period. Although patients treated with MTH had lower cell-free DNA levels at 24 hours and MTH was associated with better outcomes (lower mortality of treated patients), that difference was not significant, due to low number of patients included.

The association of plasma cell-free DNA with the outcome after cardiac arrest has been reported in two recent papers. In the first paper by Arnalich and colleagues [20], circulating DNA quantity at admission was associated with 24-hour mortality and hospital mortality. Huang and colleagues [19] quantified cell-free DNA within 2 hours from admission and at 24 hours and 72 hours after admission .They found only admission cell-free DNA quantities to be significantly different in 24-hour and hospital survivors and non-survivors. Most recently, Arnalich's group reported that mitochondrial DNA quantified within 2 hours after recovery from arrest is better in predicting 3-day survival compared to cellular circulating DNA [23].

The major novelty in the present study is superior prognostic value for hospital survival of cell-free DNA quantified 24 hours after admission than at admission. Huang's study found differing results, but included fewer patients. Also, the application of MTH is not reported in Huang's study. We found therapeutic hypothermia to be associated with dynamics of cell-free DNA quantity and, despite the fact that it showed only trend in association with survival, we believe that it is partly responsible for better prognostic value of cell free DNA at 24 hours than at admission.

Our study is the first to report correlation between free DNA quantity and cerebral performance category score, an outcome measure recommended to be used besides survival in evaluating outcomes after cardiac arrest.

The influence of an outcome predictor after resuscitation from cardiac arrest will mostly depend on current practice. It has been established that many patients in UK are declared "do not attempt resuscitation" and care is withdrawn early in the post-arrest period, when proper prognostication is not possible [24, 25]. Positive prognostic signal from a marker such as circulating DNA could reduce perception of likely adverse outcome, giving the patient a greater chance of recovery. Oppositely, withdrawal of treatment and organ donation decisions could be better substantiated with an accurate prognostic marker.

The present study was prospective and multi-centre, conducted according to strict procedures and data were collected according to the Utstein protocol. Nonetheless, the study has potential weaknesses. The most obvious limitation is the small sample size, resulting in model instability in multivariate analyses and relatively weak numbers in ROC curve analyses. Larger studies will have to be performed to address these issues. Blood sampling and specimen handling is very important factor in ensuring quality results [26], since DNA from sample leucocytes can influence the results [27]. We have invested additional efforts to ensure optimized sampling and handling in all centres, but some differences may still have occurred. The method used for DNA quantification is in principle the same as the methods other authors used in clinical research (real time PCR) but there are some differences. The gene that is used to quantify DNA is different: most authors report using β-globin gene. Also, our method reports DNA quantities in ng/μL, instead in genome equivalents as in most studies. Direct comparisons of results are therefore not possible, but conversion to genome equivalents can be made (1GE=6.6 pg DNA) [28].

Our results open several new questions that need to be answered in future research. The first may be the timing of cell-free DNA quantification. It is well established that harmful processes continue for as long as 72 hours after spontaneous circulation has been restored after cardiac arrest. Therefore we hypothesised that later quantification of cell-free DNA might be more sensitive for outcome, which was correct in our cohort. It is possible that dynamics of DNA quantities in serial measurements will be the most sensitive for outcome. Future research should include quantification of both cell-free and mitochondrial circulating DNA given the recent report [23]. We propose a larger study with serial measurements of cellular and mitochondrial DNA, addressing issues of pos-arrest treatment such as MTH, which could answer some of the remaining issues.

Conflict of interest
The authors declare they have no conflict of interest.

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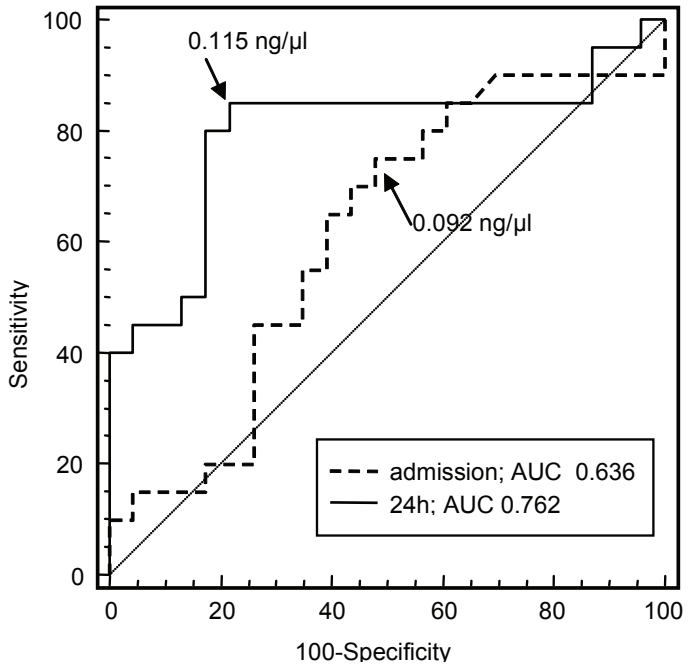
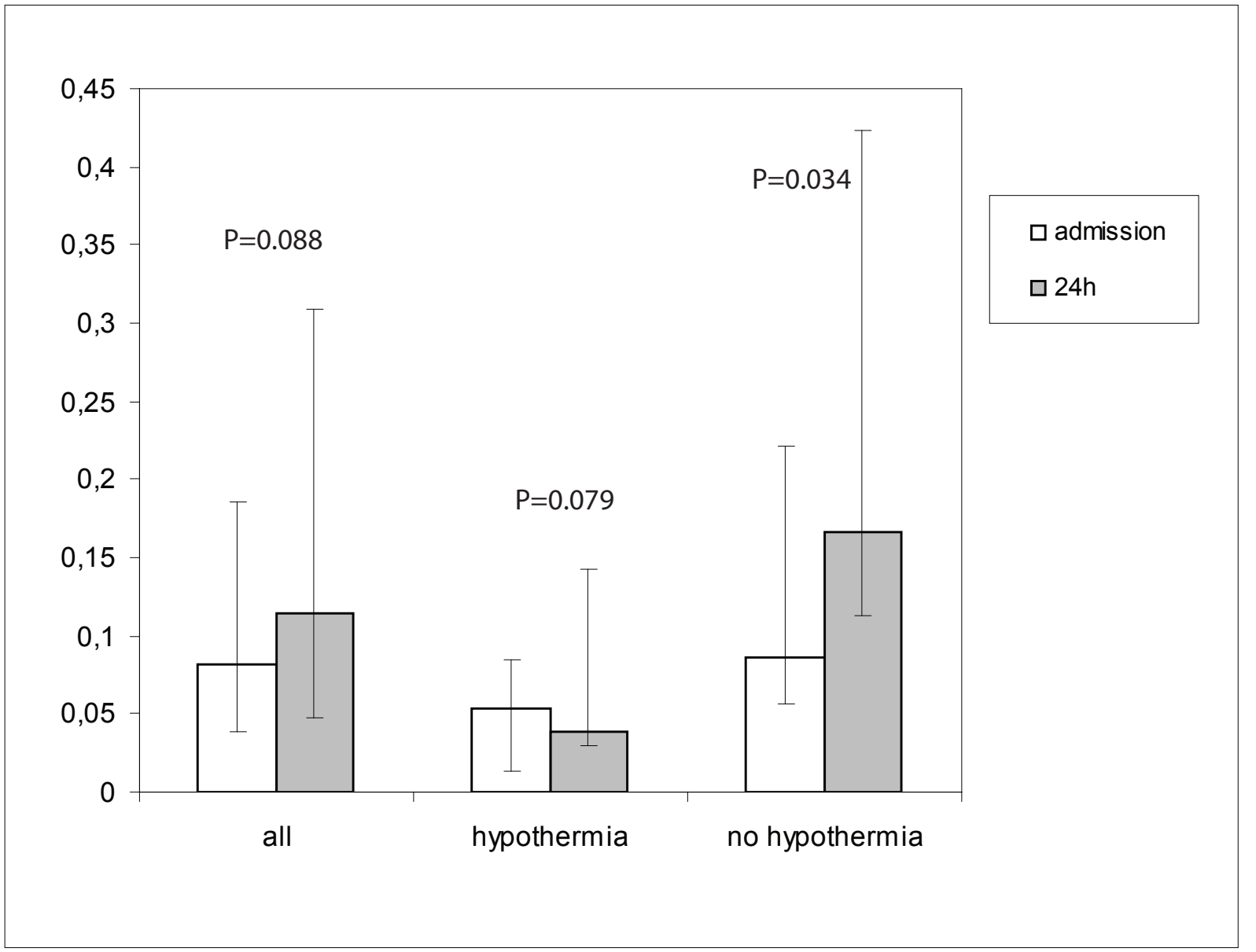
FIGURE LEGENDS

Figure 1. Receiver operating characteristic (ROC) curves for prediction of hospital mortality using cell-free DNA quantified at ICU admission (dashed line) and 24 hours after resuscitation from cardiac arrest (full line). Marked are the cut-off values with optimal sensitivity-specificity. AUC – area under ROC curve.

Figure 2. Cell free DNA quantities: at admission (white bars) and at 24 hours after admission (grey bars) for the whole cohort, patients who were and were not treated with mild therapeutic hypothermia. The height of the bar represent median, error bars represent first and third quartiles. P values from paired Wilcoxon's test.


100
80
60
40
20
0
Sensitivity
0.115 ng/µl
0.092 ng/µl
admission; AUC 0.636
24h; AUC 0.762
0
20
40
60
80
100
100-Specificity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P=0.088
P=0.079
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admission
24h
all
hypothermia
no hypothermia

Table 1. Description of patients included in the study and association of different variables with survival at hospital discharge

	Full cohort (N=69)	At hospital discharge		P
		Survivors (N=21)	Non-survivors (N=48)	
Age (years)				
Female sex	28 (40.6%)	8 (38.1%)	28 (41.7%)	0.781
Comorbidities				
- None	4 (5.8%)	2 (9.6%)	2 (4.2%)	0.751
- Hypertension	38 (55.1%)	11 (52.4%)	27 (56.3%)	0.766
- Coronary disease	20 (29%)	8 (38.1%)	12 (25%)	0.270
- Chronic heart failure	17 (24.6%)	3 (14.2%)	14 (29.2%)	0.187
- COPD	18 (26.1%)	5 (23.8%)	13 (27.1%)	0.989
- Diabetes	22 (31.9%)	5 (23.8%)	17 (35.4%)	0.502
- Obesity	22 (31.9%)	7 (33.3%)	15 (31.3%)	0.913
Type of arrest				
- cardiac in origin	42 (60.9%)	15 (71.4%)	27 (56.3%)	0.102
- respiratory	24 (34.8%)	4 (19%)	20 (41.6%)	
- other (i.e. metabolic)	3 (4.3%)	2 (9.6%)	1 (4.2%)	
Initial rhythm				
- Ventricular fibrillation	13 (18.8%)	5 (23.8%)	8 (16.7%)	0.382
- Pulseless electrical activity	21 (30.4%)	8 (38.1%)	13 (27.1%)	
- Asystole	35 (50.7%)	8 (38.1%)	27 (56.3%)	
Resuscitation				
- Time to resuscitation (“no-flow”, min)	4 (2-6)	4 (1.75-5.25)	4 (3-6)	0.209
- Resuscitation time (“low-flow”, min)	26 (20-37)	20 (13.75-23-25)	30 (23.5-39)	**<0.001**
- Witnessed arrest	42 (60.9%)	14 (29.2%)	28 (58.3%)	0.514
- Bystander CPR	17 (24.6%)	7 (33.3%)	10 (20.8%)	0.268
- CPR at admission to ED	39 (56.5%)	7 (33.3%)	32 (68.8%)	**0.021**
- Defibrillation	29 (42%)	10 (47.6%)	19 (39.6%)	0.534
Treatment				
- comatose at ICU admission	63 (91.3%)	18 (85.7%)	45 (93.8%)	0.276
- Mild therapeutic hypothermia	32 (46.4%)	12 (57.1%)	20 (41.7%)	0.236
- Acute myocardial infarction	33 (47.8%)	13 (59.1%)	20 (41.7%)	0.121
- Coronary angiography	27 (39.1%)	11 (52.4%)	16 (33.3%)	0.136
- Coronary intervention	17 (24.6%)	8 (38.1%)	7 (14.6%)	**0.029**
- Cardiogenic shock	13 (18.8%)	3 (14.2%)	10 (20.8%)	0.522
- Intra-aortic balloon pump	2 (2.9%)	-	2 (4.2%)	0.865
Cell free DNA (ng/µL)				
- admission to ICU	0.093 (0.038-0.185)	0.057 (0.023-0.145)	0.108 (0.048-0.210)	**0.048**
- 24 hours after admission	0.117 (0.037-0.309)	0.042 (0.021-0.102)	0.188 (0.121-0.388)	**0.003**

Values are medians with inter-quartile ranges or absolute and relative frequencies.
COPD – Chronic obstructive pulmonary disease; CPR – cardiopulmonary resuscitation: ED – emergency department; ICU – intensive care unit

Table 2. Multivariate analysis of factors associated with 24-hour survival.
Included are variables that were associated with 24-hour mortality in univariate analyses and showed no inter-variable correlation.

Variable	**O.R. (95% CI)**	**P**
"No-flow" time (for each additional minute)	0.69 (0.54 – 0.89)	**0.004**
Initial rhythm	0.29 (0.12 – 0.74)	**0.009**
Cell free DNA at ICU admission (ng/μL)	1.52 (0.02 – 108.05)	0.826

Table 3. Multivariate analysis of factors associated with hospital survival

Variable	O.R. (95% CI)	P
Cell free DNA at ICU admission <0.092 ng/μL	6.21 (0.59 – 65.24)	0.128
Cell free DNA 24h after admission <0.115 ng/μL	72.81 (3.76 – 1411.86)	**0.0046**
Coronary intervention	4.58 (0.25 – 81-46)	0.300
"Low-flow" time (for each additional minute)	0.80 (0.67 – 0.96)	**0.019**