The effect of fluid resuscitation and antioxidant treatment on the burn trauma induced inflammation and oxidative stress

Ph.D. thesis

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1. Abbreviations

ADH - antidiuretic hormone; **ANP** - atrial natriuretic peptide; **BBS** - burnt body surface; **BSA** - body surface area; **CAT** - catalase; **CI** - cardiac index; **CI_v** - confidence interval; **CL** - chemiluminescense; **CO** - cardiac output; **CD** - cluster of designation/ differentiation; **ELISA** - enzyme-linked immunosorbent assay; **GSH** - reduced gluthation; **H_2O_2** - hydrogen peroxide; **HMGB1** - high mobility group box protein-1; **HUO** - hourly urine output; **ICU** - intensive care unit; **IL** - interleukin; **ITBVI** - intrathoracic blood volume index; **LR** - lactated Ringer solution; **IQR** - interquartile range; **MAP** - mean arterial pressure; **MDA** - malondialdehyde; **MOD** - multiple organ dysfunction; **MODS** - multiple organ dysfunction score; **MPO** - myeloperoxidase; **NAC** - N-acetylcysteine; **NF-κB** - nuclear factor kappa-light-chain-enhancer of activated B cells; **OR** - odds ratio; **PF** - Parkland formula; **PMA** - phorbol-12 myristate-13 acetate; **PSH** - protein sulfhydryl groups; **ROC** - receiver operating characteristic; **ROS** - reactive oxygen species; **$ScvO_2$** - oxygen saturation of the central venous hemoglobin; **SH** - sulfhydryl; **SIRS** - systemic inflammatory response syndrome; **SOD** - superoxide dismutase; **SOFA** - sequential organ failure assessment; **Tx-A_2** - thromboxane-A_2; **TH1** - T-helper type 1; **TNFα** - tumor necrosis factor α; **WBC** - white blood cell.

2. Introduction

Burn trauma is caused by a wide variety of substances and external sources such as exposure to chemicals, friction, electricity, radiation, heat. Burn trauma causes usually moderate injury on the skin; it heals without scars, but special areas could also be affected like the mouth, throat or the airways. Two main factors define burn severity: depth of burn injury (which depends on the temperature and exposition time) and burnt body surface (BBS). Burn injury, affecting more than 20% of the body surface area (BSA) can lead to burn disease. This state requires special intensive care, because not only the thermally injured skin and the underlying anatomical structures are affected, but there are some pathophysiological changes that influence the whole body. Burn injury comes with severe pain. The balance of the neuroendocrine system is disturbed, consequently contrainsular hormone levels grow, hypothalamo-hypophyseal-adrenocortical system activates therefore catabolic metabolism dominates. Immunodeficiency can develop because of the reduced immunoglobulin synthesis (down-regulation), with consequence of an increased acquisition of infection. Renal vasoconstriction occurs; therefore glomerular filtration rate decreases, and haemoglobin and myoglobin, which were discharged on the ground of the thermal injury, may precipitate at the renal tubules. A common consequence is acute renal failure. Adaptive reactions come into action to restore the circulating intravascular volume, the secretion of ADH increases while the plasma level of ANP decreases. Gastrointestinal vasoconstriction occurs due to Tx-A_2 release. Circulation in mesenteric blood vessels lessens, therefore gut mucosal barrier becomes damaged which leads to increased bacterial and endotoxin translocation to the circulation. The immune system activates a high amount of inflammatory mediator release. Macrophage and leukocyte activation triggers free radical, arachidonic acid and metabolites formation which play a role in early edema formation and cytokine (TNFα, IL-1, -2, -6) production. The released metabolites have significant effect on both local wound- and systemic inflammatory reaction. After injury - almost instantly - increases the capillary permeability, vasodilatation appears in which histamine, serotonine, bradykinin, prostaglandins, leukotriene, proinflammatory cytokines and free radicals play a role. Increased blood vessel permeability leads to fluid and protein flux into the interstitium. On the basis of this mechanism, at a certain extent of burns i.e. more than the 20% of the BSA, generalised edema formation occurs; this fluid loss leads to hypovolaemia in the intravascular space and to hypoperfusion which subsequently results in the damage of cells and organs.

3. The aim of our studies

Oxidative stress and systemic inflammatory response syndrome (SIRS) play an important role in edema formation, causing severe hypovolemia following burns. A more adequate fluid resuscitation regime guided by intrathoracic blood volume index (ITBVI) might beneficially modulate the inflammatory processes following burn injury. The other possibility is to reduce fluid requirement of the burned patients via influence on the underlying pathophysiological processes of edema formation. However, only few data exists regarding the effect of the antioxidant therapy in patients suffering from burn injury. The role of oxidative stress markers, different citokines and leukocyte cell surface markers were well studied in different clinical aspects, but the time course and the kinetic of changes in oxidative stress markers and inflammatory or anti-inflammatory cytokines as well as their prognostic value are not well cleared. The aims of our work were the following:

1. We wanted to follow up the time course of pro- and anti-inflammatory cytokine and plasma high mobility group box protein-1 (HMGB1) levels in the immediate postinjury period to investigate their prognostic value in patients with severe burn injury.

2. Fluid resuscitation management can influence inflammatory response after burn injury. We aimed to analyze the effects of two different fluid resuscitation methods on the cytokine production and expression of the leukocyte surface markers. Our objective was to compare the effect of ITBVI and hourly urine output (HUO)-guided fluid therapy on the stimulated and non-stimulated plasma levels of pro- and anti-inflammatory cytokines and on the expression of different adhesions molecules.

3. We also wanted to compare the oxidative stress parameters, pro- and antiinflammatory cytokines and expression of leukocyte adhesion molecules in patients receiving N-acetylcysteine (NAC) treatment and in standard care without NAC supplementation. We aimed to assess the differences in organ function scores (multiple organ dysfunction (MOD) score and sequential organ failure assessment (SOFA)) and to compare the vasoactive drug and fluid requirement in patients receiving NAC and in standard care.

4. Patients and methods

4.1. Patients

After receiving permission from the local ethics committee the patients or nearest relative provided a written, informed consent. After randomisation (with closed envelope method) the patients were divided into the study groups.

Inclusion criteria: Inclusion criteria were flame burn injury affecting more than 15% of the BSA, necessity for mechanical ventilation, and admission to our ward within 3 hours after injury. *Exclusion criteria:* Exclusion criteria were electrical injury, presence of any obvious bacterial infection on admission, extreme burn severity (>80% BBS or Baux index>120), previously documented chronic left heart or renal insufficiency, age younger than 18 years, documented haematological disease in the past medical history, previous medication affecting the inflammatory response of the body to burn injury (e.g. chronic use of corticosteroids, cytostatic treatment in the last 30 days), or absence of consent to the study.

Patient treatment protocol: All of our patients required immediate intensive care unit (ICU) treatment. Patients were treated in a uniform way and practice patterns were not changed. If inhalation injury was suspected bronchoscopy was carried out for verification. Excision and grafting were started within 72 hours. 20-30% of BSA was excited and grafted in one sitting. Operations were repeated in every 3-4 days. Enteral feeding was commenced on the first day after injury when hemodynamic stability was reached. All patients were mechanically ventilated after admission and every patient survived the first week. Tracheostomy was performed before the first grafting to avoid complications due to coagulopathy.

4.2. Methods

4.2.1. Fluid resuscitation protocol and monitoring

At the beginning of our clinical research compared the effect of fluid resuscitation methods guided by HUO and ITBVI on the inflammation markers after burn trauma. In the later studies fluid resuscitation was guided by invasive transpulmonary thermodilution hemodynamic measurements and the target parameter was the ITBVI.

Intravenous fluid resuscitation was guided by urine output monitoring in the HUO group and by invasive hemodynamic monitoring in the ITBVI group. In both groups for the invasive transcardiopulmonary hemodynamic measurements a special arterial catheter (PiCCO, Pulsion Medical Systems, Munich, Germany), and a special probe to record the oxygen saturation of central venous hemoglobin ($ScvO_2$) (CeVOX, Pulsion Medical Systems, Munich, Germany) was inserted via the central venous catheter. The initial infusion rate for the first 24 hours was set according to the Parkland formula (PF) (4 ml kg^{-1} BBS^{-1}) in both groups. The initial infusion rate was set to provide half of the calculated first day volume within the first 8 hours time. Only lactated Ringer (LR) solution (BBraun Melsungen AG, Melsungen, Germany) was used for intravenous fluid replacement in the first 24 hours.

HUO group: if the average urine output was lower than 0.5 ml kg^{-1} h^{-1} for at least 2 h, the intravenous infusion rate was increased by 0.05 ml kg^{-1} h^{-1} for the next 2 h. The infusion rate was decreased by 0.05 ml kg^{-1} h^{-1} if the average urine output exceeded 1.0 ml kg^{-1} h^{-1} for at least 2 consecutive hours. If the intravenous fluid replacement regimen had failed to maintain a mean arterial pressure (MAP) above 70 mmHg, norepinephrine infusion was used, with a maximum rate of 0.1 μg kg^{-1} min^{-1}. The attending physician was blinded to the results of invasive hemodynamic monitoring in the HUO group. Invasive hemodynamic measurements were performed 8 hourly for the first 3 days after injury.

ITBVI group: invasive transpulmonary thermodilution hemodynamic measurements were performed in every 2 h by the same method as in the HUO group. The goal of resuscitation was to maintain ITBVI between 800 and 850 ml m^{-2}. If ITBVI was under 800 ml m^{-2}, the infusion rate was increased by 10%. If ITBVI was under 750 ml m^{-2}, 500 ml LR was administered as an intravenous bolus, and the hemodynamic measurements were repeated. LR solution was administered until the targeted value was reached. If ITBVI was over 850 ml m^{-2}, the infusion rate was decreased by 10%. If the target range of ITBVI had been reached but oliguria (diuresis<0.5 ml kg^{-1} h^{-1}) and/or hypotension (MAP<70 mmHg) were present, a norepinephrine administration was initiated on the basis of hemodynamic monitoring with a maximum rate of 0.1 μg kg^{-1} min^{-1}; and dobutamine was administered in case $ScvO_2$ was lower than 70%.

Both groups had, on days 2-6, background intravenous fluid replacement at 2 ml kg^{-1} h^{-1} using balanced salt solutions topped with LR and hydroxyethyl starch (Voluven; Fresenius AG, Frankfurt, Germany) infusions, according to HUO or ITBVI.

4.2.2. Scoring system for inotrop and vasopressor drug administration

For assessment of inotrope and vasopressor administration a scoring system has been developed by our group. The inotrope/vasopressor requirement of the patients was assessed hourly during the study period. Patients were assigned into no drug, low dose or high dose subgroups. The daily score was calculated by summing all hourly values. The cut off value between low and high dose groups regarding norepinephrine was 10 μg kg^{-1} h^{-1}, while regarding dobutamine; it was 0.3 mg kg^{-1} h^{-1} (Table 1.).

Table 1.: Scoring system for inotropic and vasopressor drug administration

Drug	No-drug	Low dose	High dose
Norepinephrine	0	1	2
Dobutamine	0	1	2

4.2.3. Clinical scoring systems

MOD and SOFA scores were allocated. These scores were calculated in each patient daily after admission during the stay in ICU. MOD score was constructed for the assessment of dysfunction of six vital organ systems using simple physiologic measures. It correlates strongly with the risk of ICU and hospital mortality and generally accepted as a composite marker of severity of condition that involves therapeutic effects in ICU. SOFA score was designed to describe the sequence of complications in the critically ill patient. It is not suitable to predict outcome and can be calculated by scoring the worst daily values of six organs.

4.2.4. NAC supplementation

In the NAC group the standard treatment was supplemented with administration of NAC (Fluimucil 100 mg ml^{-1}, Zambon Group S.p.A., Bresso, Italy) as a bolus of 150 mg kg^{-1} followed by a continuous administration of 12 mg kg^{-1} h^{-1} for the next 5 days.

4.2.5. Measurements and laboratory techniques

4.2.5.1. Blood sampling and analysis

Acute phase reaction usually lasts for 3 days; therefore we had presumed that 6 days period would open a wide time window that could be enough for detecting both the uprising and descending immunological phases. Venous blood samples were taken at the time of hospital admission (day 1) and in 5 consecutive days at 7 o'clock a.m. (days 2-6) thereafter. The first samples were taken 3.7 h (IQR, 3.2-4.2) after burn injury. Blood samples were taken always before operations or painful dressing changes, samples from healthy volunteers (n=9) were used as control. Blood was taken on a single day and the values were repeatedly used as controls throughout six days time for statistical purposes. Reference population was matched to age and sex.

4.2.5.2. Biochemical assays

All of the samples were transferred on 4°C and processed in 6 hours after takeoff.

4.2.5.2.1. Measurement of pro-, and antioxidant parameters:

Measurement of malondialdehyde (MDA) with Ohakawa method: the plasma MDA is one of the derivatives originating from oxidative damage of poly-unsaturated fatty acids, thus indirectly shows intensity of lipidperoxidation due to oxidative stress. We used tetrametoxipropane as a standard and MDA was expressed in μM/l.

Determination of reactive oxygen species (ROS) production in whole blood: activated leukocytes, mainly neutrophils, are potential sources of ROS during inflammation. The lag phase between PMA stimulation and the start of steep elevation in radical production was also determined.

Measurement of plasma myeloperoxidase (MPO) activity: MPO is a lysosomal enzyme that is found in neutrophil granulocytes and its plasma level elevates during inflammation.

Measurement of reduced gluthation (GSH) in whole blood: it is a basic endogenous antioxidant, the level of which is reduced due to oxidative stress of various origins. Using a standard GSH series for calibration, values were expressed in μM/l.

Measurement of plasma plasma sulphydryl groups (PSH) level with Ellman's reagent: plasma SH originates predominantly from plasma proteins and participates in the defence against oxidative stress. GSH standard series were used for calibration. The PSH amount was expressed in μM/l.

Determination of superoxide dismutase (SOD, mainly Cu/Zn-SOD) enzyme activity in whole blood: SOD is an enzymatic endogenous antioxidant which catalyses the dismutation of the superoxide free radical. The values of SOD enzyme activity were given in IU/ml.

Determination of catalase (CAT) enzyme activity in whole blood: CAT enzyme activity in hemolysate was determined by the Aebi method. CAT metabolizes hydrogen peroxide (H_2O_2) by reducing it to water and oxygen. This prevents the second generation of toxic intermediates. The values of CAT enzyme activity were given in BU/ml.

4.2.5.2.2. *Cytokine measurements:* Concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNFα were measured by the Cytometric Bead Array Human Inflammation Kit (BD Biosciences, USA) according to the manufacturer's instructions both in native plasma samples and from plasma samples separated after in vitro stimulation of whole blood with PMA for 4 hours at 37 °C. FACS Calibur (BD Biosciences, USA) flow cytometer was used for acquisition of data.

4.2.5.2.3. HMGB1: Plasma concentration of HMGB1 was measured by a commercially available HMGB1 enzyme-linked immunosorbent assay (ELISA) kit (Shino-Test Corporation, Kanagawa, Japan) according to the manufacturer's instruction.

4.2.5.2.4. Measurement of leukocyte cell surface markers: Flow cytometry was used to analyze the adhesion molecules (CD11a, CD11b, CD18, and CD49d), lipopolysaccharide receptor CD14, and leukocyte activation marker CD97 expression on leukocytes. Cell immunofluorescence and light scatter data were acquired on a FACS-Calibur (BD Biosciences, San Jose, CA) flow cytometer and analyzed by Cellquest software.

4.6. Statistical analysis

Multi measure ANOVA and Kruskal-Wallis rank sum test was used for intergroup analyses and testing differences within groups at different time points. Data were analyzed in univariate and multivariate logistic regression models and Fischer's exact test was also performed. In this manuscript Mann-Whitney test was used to compare the values of the two groups. The receiver operating characteristic (ROC) analysis was used for assessment of specificity and sensitivity of the data regarding mortality. Data were expressed as median and interquartile range (IQR) (standard 25th-75th percentile). Odds ratio (OR), 95% confidence interval (95% CI_v), and p values were calculated. Values of $p<0.05$ were considered significant.

5. Results

5.1. Time course of pro- and anti-inflammatory cytokine and HMGB1 levels in patients with burns

39 patients were involved in the study. Based on the clinical outcome, patients were divided into non-survivor- and survivor-groups. In the non-survivor group patients were significantly older, occurrence of sepsis was significantly higher and ICU length of stay was significantly shorter. The onset of sepsis was on the ninth (IQR, 7-12) day. Hospital mortality rate was 46%. 11 of 18 patients died of septic complication in the ICU.

Changes in cytokines in non-stimulated plasma samples

The average values of IL-1β, IL-12p70, and TNFα concentrations were below the detection limit of the assay (IL-1β: 7.2 pg ml^{-1}, IL-12p70: 1.9 pg ml^{-1}, TNFα: 3.7 pg ml^{-1}) during the study period. IL-6, IL-8, IL-10 concentrations were higher than the normal values in human plasma. Pro-inflammatory cytokines IL-6 and IL-8 were only moderately elevated

on admission and started to increase markedly from day 2 reaching the peak values on day 3. The measured cytokine levels compared to day 1 were significantly ($p<0.05$) higher during the whole study period. In contrast to pro-inflammatory cytokines IL-10 concentrations were markedly elevated at the time of hospital admission and gradually decreased thereafter. Mean levels of IL-10 were significantly ($p<0.05$) lower on days 3-6 compared to day 1. IL-6/IL-10 ratios showed elevation reaching the peak value on day 4. The calculated values were significantly ($p<0.01$) higher during the study period compared to day 1. IL-8/IL-10 ratios were elevated from day 1 reaching the peak values on day 4. Ratios calculated during the whole study period were significantly ($p<0.05$) higher than the ratio calculated on admission.

Changes in cytokines in stimulated plasma samples

After PMA stimulation IL-12p70 remained undetectable. IL-6 levels showed a moderate elevation compared to non-stimulated samples. The peak value was reached on day 4. The levels on days 2-4 were significantly ($p<0.05$) higher than measured on admission. IL-8 showed a marked elevation after PMA stimulation. It reached the peak value on day 5. The levels on days 5-6 were significantly ($p<0.05$) higher than on day 1. IL-1β became detectable after PMA stimulation. It reached the peak value on day 3 without any significant difference during the study period. TNFα became detectable after PMA stimulation similarly to IL-1β levels. Significant difference was not found during the study period. The peak value was reached on day 3. IL-10 showed only a moderate elevation after PMA stimulation. IL-10 concentrations gradually decreased after admission. IL-10 levels on days 4-6 were significantly ($p<0.05$) lower than on day 1. IL-6/IL-10 ratios showed an increasing tendency reaching the peak value on day 4. Significant ($p<0.05$) difference could be observed between day 1 and days 2-6. IL-8/IL-10 ratios increased during the study period. The highest value was detected on day 6. Significant ($p<0.05$) difference were between day 1 and days 3-6. IL-1β/IL-10 ratios reached the peak value on day 2. A decreasing tendency was observed thereafter. The ratio measured on day 6 was significantly ($p<0.05$) lower than that measured on admission. TNFα/IL-10 ratios remained nearly on the same level during the study period without any significant difference. Comparing IL levels and ratios in blood samples taken on the morning of the operation and on the next day significant differences were not found.

Changes in cytokines in non-stimulated plasma samples of survivor and non-survival patients

IL-6, IL-8, IL-10 concentrations were higher in both survivors and non-survivors groups than the normal values in human plasma throughout observation. Pro-inflammatory cytokines IL-6 and IL-8 were only moderately elevated on admission and started to increase markedly from day 2. IL-6 reached the peak value in non-survivors on day 4 and a moderate decrease could be found after this time. In survivors it peaked on day 2, and remained on a lower, but elevated level thereafter. Significant differences were found between survivors and non-survivors on day 4 ($p<0.05$) and day 5 ($p<0.001$). IL-8 showed a marked elevation after admission reaching the peak value in survivors on day 3, in non-survivors on day 4. After reaching the peak value, IL-8 level started to normalise in survivors, whereas in non-survivors a plateau phase could be observed thereafter. Significant differences were found between survivors and non-survivors on day 4 ($p<0.05$), day 5 ($p<0.01$) and day 6 ($p<0.05$). IL-10 concentrations were markedly elevated at the time of hospital admission in the non-survivors and gradually decreased thereafter, whereas in survivors it showed only a moderate elevation. Mean levels of IL-10 in non-survivors were significantly higher compared to survivors during the whole study period ((day 1 ($p<0.001$), day 2 ($p<0.05$), day 3 ($p<0.05$), day 4 ($p<0.01$), day 5 ($p<0.001$) and day 6 ($p<0.05$)). IL-6/IL-10 ratios showed elevation in both in survivor and non-survivor patients. It reached the peak value in survivors on day 4, in non-survivors on day 5. Significant difference could be observed on admission with higher level in the survivors

($p<0.01$). IL-8/IL-10 ratios were elevated in both groups of patients from day 1 showing a significant difference between groups on day 1 ($p<0.01$). It reached the peak values in survivors on day 3 in non-survivors on day 6.

Changes in cytokines in stimulated plasma samples of survivor and non-survivor patients

IL-6 reached the peak value both in survivor and non-survivor patients on day 4. There was no significant difference between groups similarly to IL-8, which reached the peak value in survivors on day 5 in non-survivors on day 6. IL-1β showed a more marked elevation in survivors than in non-survivors showing significant differences between groups on day 2 ($p<0.05$) and day 3 ($p<0.05$). It reached the peak value in survivors on day 3 while it remained on a slightly elevated level in non-survivors during the whole study period. TNFα levels were more markedly elevated in survivors than in non-survivors. It reached the peak value in survivors on day 3 in non-survivors on day 4. Significant differences between groups could be observed on day 1 ($p<0.05$), day 2 ($p<0.05$) and day 3 ($p<0.05$). IL-10 concentrations were markedly elevated at the time of hospital admission in non-survivors and gradually decreased thereafter, whereas in survivors it showed only a moderate elevation similarly to non-stimulated samples. Mean levels of IL-10 in non-survivors were significantly higher compared to survivors from day 1 to day 5 ((day 1 ($p<0.001$), day 2 ($p<0.05$), day 3 ($p<0.05$), day 4 ($p<0.05$) and day 5 ($p<0.05$)). IL-6/IL-10 ratios showed elevation in both in survivor and non-survivor patients. It reached the peak value in survivors on day 3, in non-survivors on day 5. Significant difference could be observed on admission with higher levels in survivors ($p<0.05$). IL-8/IL-10 ratios were elevated in both groups of patients during the whole study period showing significantly higher levels in survivors ((day 1 ($p<0.05$), day 2 ($p<0.05$), day 3 ($p<0.001$), day 4 ($p<0.05$), day 5 ($p<0.05$) and day 6 ($p<0.05$)). The peak value was reached in survivors on day 5 whereas in non-survivors on day 6. IL-1β/IL-10 ratios were elevated in both groups with peak values in survivors on day 2 and non-survivors on day 4. Statistical differences were not found between groups. TNFα/IL-10 ratios were significantly higher in survivors on day 1 ($p<0.01$), day 2 ($p<0.05$) and day 3 ($p<0.05$). It reached the peak value in survivors on day 3 whereas it remained on a slightly elevated level in non-survivors.

Changes in plasma HMGB1 concentration

Plasma HMGB1 concentration was markedly elevated on hospital admission in both survivors and non-survivors. The difference between survivors and non-survivors was significant only on day 1. HMGB1 level significantly declined thereafter in both groups during the observation period.

Receiver operating characteristic analysis

Comparing the predictive values of different cytokines and cytokine ratios IL-10 levels in the stimulated and non-stimulated blood were the best predictor of sepsis and mortality followed by levels of IL-8 in stimulated and IL-6 and IL-8 in non-stimulated blood. Survival and sepsis were significantly predicted by HMGB1 levels on admission ($p=0.013$, OR: 1.217 [1.042-1.422]; $p=0.037$, OR, 1.131 [1.007-1.270], respectively). The number of cases was too low to get reliable statistical output using multivariate logistic regression model. The ROC analysis of data on admission showed that at the level of 14 pg ml^{-1} IL-10 predicted the lethality with 85.4% sensitivity and 84.2% specificity. HMGB1 indicated lethality at a level of 16 ng/mL, with 75.0% sensitivity and 85.7% specificity. Comparing HMGB1 values to cytokine results, a positive correlation was found between HMGB1 and IL-10 levels on admission ($r=0.746$, $p<0.01$), but a close correlation could not be found between HMGB1 level on admission and IL-6 and IL-8 levels on admission and later on.

Septic vs. nonseptic patients (*non-stimulated plasma samples)*

Comparing septic and nonseptic patients, HMGB1 levels on admission were significantly higher in septic ones only on admission. IL-10 levels in septic patients were significantly higher compared with nonseptic ones during the whole study period. There was significant difference in proinflammatory cytokine levels during our study period. Significant differences between septic and nonseptic patients in IL-6 were observed on days 4-6, respectively during our observation. There was a significant difference between septic and nonseptic patients in IL-8 on days 1, 4-6, respectively. Positive correlations ($r=0.669$, $p<0.01$) were found between burned body surface and HMGB1 concentrations on admission.

5.2. Effects of fluid resuscitation methods on the pro- and anti-inflammatory cytokines and expression of adhesion molecules after burn injury

The study population consisted of 30 patients (6 females, 24 males). There were no significant differences between groups regarding age, burn size, presence of inhalation injury and measured parameters on admission.

Fluid resuscitation, hemodynamic and clinical parameters

The fluid resuscitation algorithm was followed for each patient in both groups. Significantly more fluid was administered in the ITBVI group than in the HUO group in the first 24 hours after injury and 56% of the extra fluid was administered in the first eight hours, 29% in the second eight hours and only 15% in the last eight hours. Patients in HOU group compared to patients in ITBVI group required significantly more fluid on day 2. Significant differences were not found between groups regarding hospital mortality, hospital and ICU stay, days on ventilator, occurrence of sepsis and multiple organ failure. MOD scores calculated 48 and 72 hours after injury were significantly lower in ITBVI group compared to HUO group. Complications associated with invasive monitoring were not detected during the study period**.**

Changes in plasma cytokine levels

In non-stimulated plasma samples the average values of IL-1ß, IL-12p70, and TNFα concentrations were below the detection limit of the assay (IL-1ß: 7.2 pg ml^{-1}, IL-12p70: 1.9 pg ml^{-1}, TNFα: 3.7 pg ml^{-1}) during the study period. IL-6, IL-8, IL-10 levels did not show significant differences between ITBVI and HUO groups on admission. IL-6 levels on days 2-3 were significantly higher in the ITBVI group ($p<0.05$) whereas elevated levels of IL-10 could be observed in the HUO group on days 4-6 ($p<0.05$). The IL-6/IL-10 ratio on days 2-3, and the IL-8/IL-10 ratio on days 3-5 were significantly higher in the ITBVI group ($p<0.05$).

In stimulated samples the levels of the studied cytokines except IL-12p70 were above the detection limit. The value of the different cytokines did not differ significantly between groups on admission and significant differences could not be observed in the pro-inflammatory cytokine levels but significant differences in IL-10 levels were observed on days 4-5 ($p<0.05$) with higher levels in the HUO group.

Changes in adhesion molecules

The granulocyte CD11a levels were significantly higher on the second day in the HUO group ($p<0.05$) compared to the ITBVI group. CD11b levels were significantly higher in the HUO group on days 4-6 ($p<0.05$) than in the ITBVI group. There were no significant difference between groups regarding granulocyte CD49d and CD97. Lymphocyte CD11a on days 5-6, lymphocyte CD11b on days 3-6, lymphocyte CD49d on days 2-6 and lymphocyte CD97 on day 6 were significantly lower ($p<0.05$) in the ITBVI group than in the HUO group whereas CD18 did not show significant difference between groups. Comparing the HUO group to the ITBVI group, monocyte CD11a, CD11b, CD18 levels showed a significant

decrease ($p<0.05$) in ITBVI group on days 4-6. The CD14 level was significantly lower in the ITBVI than in the HUO group on days 3-5 ($p<0.05$), whereas CD49d and CD97 did not differ significantly between groups.

5.3. The effect of NAC treatment on the oxidative stress, expression of leukocyte surface markers and pro- and anti-inflammatory cytokines after burn injury

Demographic and initial data

30 patients were involved in this prospective randomised study. There were no significant differences between groups regarding age, BSA, extent of deep burn, occurrence of inhalation injury, mechanism of burn and in calculated organ function scores.

Changes in leukocytes

White blood cell count was markedly elevated in both patient groups, and decreased during the study period reaching the level of significance from day 3 without any differences between groups. Burn trauma induced acute severe granulocytosis and lymphocytopenia. The relative number of granulocytes decreased, the relative number of lymphocytes increased from day 2, and granulocyte ratio was lowest and that of the lymphocyte ratio was highest on days 3-4 in both groups without significant differences between groups. Opposite to NAC treatment, in patients with standard therapy an increasing tendency in granulocyte ratio, and a decreasing tendency in lymphocyte ratio was observed from day 3. The relative number of monocytes increased significantly on day 5 in both groups.

Changes in oxidative stress markers

Pro-oxidant markers

Plasma MDA level increased significantly on day 2 in both groups, without any significant differences between groups. Plasma MPO activity increased on days 5-6 in both groups but it was not significant. No significant differences were found between groups. Maximal value and rate of ROS production showed significant elevation in both groups from day 4 compared to the day 1 values, without any significant differences between groups.

Endogenous antioxidants

GSH in hemolysate was higher in both groups compared to the values in healthy volunteers on admission, and decreased significantly in both groups from day 2. GSH level was significantly higher in NAC treated group compared to standard therapy on days 4-6. Plasma SH level decreased moderately in NAC group showing significant differences from day 4, whereas it showed a marked decrease in standard group from day 2. Differences in PSH levels were significant between groups from day 2. SOD activity in hemolysate was moderately decreased compared to the values of healthy volunteers, and unchanged during study period without any significant differences between groups. CAT activity in hemolysate was significantly increased compared to the values of healthy volunteers, and it remained unchanged during the observation period in standard group, and showed a slight, but not significant elevation in NAC group, without any significant differences between groups.

Changes in cytokine levels in native plasma

IL-6 showed a significant elevation from day 2 in standard group and from day 3 in NAC treated patients compared to day 1. Significant differences could be found between groups on days 4-5. Serum IL-8 levels were elevated on days 2-6 in standard group and on days 3-4 in NAC group. The groups differed significantly on days 4-6. IL-10 showed only a slight decreasing tendency in standard group and a more pronounced decrease in NAC treated

group reaching the level of significance from day 3. Significant differences could be found between groups on days 4-6.

Changes in leukocyte cell surface markers

Granulocyte CD11a levels were significantly higher on days 4-6, compared to day 1 in standard group. CD11a levels showed only a moderate elevation in the NAC group, without any significant differences compared to day 1. Significant differences between groups could be observed on days 4-6. CD11b showed a slight decreasing tendency from day 5, without any significant difference between groups or compared to day 1. CD18 elevated significantly in standard group from day 2, whereas it showed only a slight increasing tendency on days 3-5 in NAC group. Significant differences could be observed between groups on days 4-6. CD49d levels were significantly higher in both groups from day 3 during the whole study period, but significant difference could be found between groups on day 2 only. CD97 levels showed a more marked elevation in standard group, with significant higher levels on days 3-6. The elevation in NAC group was significant on day 6 only. The differences between groups were significant on days 2-6.

Lymphocyte CD11a levels showed a moderate increasing tendency in the standard group and a slight decreasing tendency in NAC group, failing to show any significant differences compared to day 1 in both groups. Significantly higher levels of CD11a could be found in standard group compared to NAC group on days 3-6. CD11b didn't show significant changes, either in standard or in NAC group, or significant differences between groups. CD18 levels decreased in both groups, although more markedly in NAC group, without significant differences compared to day 1 and between groups. CD49d levels showed elevation with significantly higher levels on days 4-6 in standard group, where as its levels remained unchanged in NAC group. Significant differences were found between groups on days 3-6. CD97 showed an increasing tendency in both groups, which reached the level of significance in standard group on days 5 and 6, without significant differences between groups.

Monocyte CD11a failed to show any significant differences compared to day 1 or between groups, similarly to CD11b and CD18 levels. CD49d increased in both groups from day 2, and although it remained significantly elevated during the whole study period in standard group, its level decreased in NAC group from day 5. Significant differences compared to day 1 could only be observed in NAC group on days 3-4. Significant differences could be found between groups on days 4-6. CD97 elevated in standard group from day 3, whereas its levels remained nearly unchanged in NAC group. Significant differences could not be detected compared to day 1 in either group, but significantly higher levels could be observed in standard group compared to NAC group on days 3-6. CD14 showed a decreasing tendency from day 4 in both groups. Significant differences could be observed compared to day 1 in standard group on days 4-6, and in NAC group on days 5-6.

Clinical outcome parameters

NAC treatment was well tolerated. Adverse effects, apart from more pronounced sputum production not affecting the ventilator requirement were not observed during the study period. The inotrope and vasopressor requirements in patients of standard group were significantly higher on days 4-6. There were no significant differences in preload of patients reflected in ITBVI during the whole study period. Daily SOFA and MOD scores did not show any significant difference between NAC and standard group during the study period. The fluid requirement tended to be lower in the NAC group during the first 24h [(3.5 ml kg^{-1} BBS^{-1} (3.1-5.4) vs. 4.2 ml kg^{-1} BBS^{-1} (3.7-7.4)]. But this difference was not significant statistically. Significant differences could not be observed in days on respirator and ICU length of stay. Excluding non-survivors, the differences regarding necessity of mechanical ventilation and

ICU length of stay remained non significant between NAC and standard group [31 (24-42) vs. 30 (27-36) and 43 (34-59) vs. 42 (36-56); respectively]. Mortality tended to be higher in the standard group (6 vs. 4) but this difference was not statistically significant.

6. Discussion

Burn trauma induces severe oxidative stress and leukocyte activation. The role of oxidative stress markers, different citokines and leukocyte cell surface markers were widely studied in different clinical aspects, but the time course and the kinetic, as well as their prognostic value are not well clarified. We have followed the changes in proinflammatory and anti-inflammatory cytokines and HMGB1 in patients with severe burn injury on admission and for five consecutive days. Our results confirmed that an overwhelming anti-inflammatory response after burn reflected in marked elevation of IL-10 levels is associated with more frequent occurrence of sepsis and higher mortality rate. The results demonstrate an early increase in plasma HMGB1 within 5 h of burn injury in humans. In addition, we have found that shortly after burn injury, HMGB1 levels were significantly higher in septic as well in non-surviving patients and have good predictive values regarding sepsis and mortality.

The elevation of pro- and anti-inflammatory cytokines following burn has been reported by other studies. Although most of the studies report an elevation in pro- and anti-inflammatory cytokines following burn injury in our study IL-1β, TNFα and IL-12p70 in the non-stimulated samples and IL-12p70 in the stimulated samples did not reach the detection limit of the kit in our patients. Low levels of certain plasma cytokines measured in non-stimulated samples might arise from either diminished release from cytokine producing cells or fast biodegradation. In this case the determination of the whole amount of pre-synthesized cytokines in leukocytes using receptor independent stimulation may be informative about the potential sources of these cytokines. Surprisingly, we did not find elevated levels of IL-1ß and TNFα in native plasma, although most publications showed elevated levels of these cytokines immediate after injury.

IL-6, IL-8, IL-10 produced mainly by macrophages, play an important role in the initial phase of the post-burn pathophysiological processes. Yeh et al. reported elevated levels of IL-6 and IL-10 in the early period after burn supporting our results, but contrary to our study significant differences were not found between survivors and non-survivors regarding the first mean serum level of IL-6 and IL-10. Ozbalkan et al. found significant differences between survivors and non-survivors regarding serum IL-10 and IL-8 levels measured on admission similarly to our results. In our study the level of IL-10 on admission was significantly higher in non-survivors, moreover the IL-10 level on admission had prognostic value. Our results are in concordance with that of others, and confirm that an early shift can be observed towards anti-inflammatory cytokine production which makes the patient susceptible to infections. Our data showed that almost every patient who died suffered from sepsis and it was associated with significantly higher levels of IL-10 compared to survivors on admission and 2 days thereafter.

The level of IL-12p70 was not elevated during the study period. It confirms the result of Finnerty et al. who found an elevated level of IL-12p70 in burned children only from the second week after injury. O'Sullivan et al. also found that trauma and major burns led to decreased levels of IL-12 and to increased production of IL-4 and IL-10. The levels of IL-1β and TNFα were also low in our study despite the fact that they are produced by activated macrophages in the initial phase of burn injury and they might be responsible for the haemodynamic changes following burn trauma. The depressing activity of elevated level of IL-10 on the activity of NF-κB, which is essential for the synthesis of proinflammatory cytokines in TH1 cells and in macrophages, might explain the low values of pro-inflammatory cytokines TNFα, IL-1β, IL-12p70, however, it has been proven in mice that after thermal

injury macrophages are resistant to the effect of IL-10 in contrast to other illnesses. Elevated levels of TNFα, IL-1β, IL-6, IL-8 and IL-10 could be detected in the stimulated samples similarly to other studies. On the other hand the detection limits of the kits are different. The balance between pro- and anti-inflammatory cytokines may be essential for appraising the genuine effect of different cytokines.

HMGB1 was recently identified as a potent proinflammatory mediator playing an important role in the pathogenesis of human diseases including sepsis, hemorrhagic shock, mechanical trauma, surgical stress, cerebral and myocardial ischemia, and pancreatitis. Several experimental data confirm that burn injury induces an elevation in plasma HMGB1 concentration. Using the same commercially available ELISA system, plasma median HMGB1 concentrations were less than 2 ng ml^{-1} in healthy humans. Our results confirmed a marked elevation in HMGB1 after burn injury, as recently reported by Dong and associates. In our study, plasma HMGB1 levels were markedly elevated in the very early hours after burn trauma. These results suggest that in contrast to sepsis, HMGB1 release is an early event after traumatic or burn injury in humans. Moreover, HMGB1 level was significantly higher in our study both in septic and non-survivors patients than in nonseptic and survivor ones on admission; and despite the small study population, it had a predictive value. HMGB1 levels on admission correlated well with the burned body surface. Elevated level of HMGB1 could inhibit the production of proinflammatory cytokines in TH1 cells and in macrophages and could lead to T-cell immune dysfunction. Septic episodes in our patients occurred in the later phase of the treatment, so the slight elevation in plasma HMGB1 concentration at the end of the observation period might be a sign of impending sepsis, but a close correlation could not be verified.

The results clearly reveal that burn injury induces a very early HMGB1 and IL-10 release in humans, and it may have an important impact on the immune function of patients after burn trauma. Future research with a larger number of patients might further elucidate these potential relationships with HMGB1 and IL levels. The role of HMGB1 and IL-10 in post-trauma inflammation and organ dysfunction makes it a potential target for therapy directed at reducing postinjury morbidity and mortality.

In our other study we investigated the effect of the fluid resuscitation methods guided by HUO and ITBVI on the cytokine production and expression of the leukocyte surface markers. Our results demonstrates significantly higher IL-6, significantly lower IL-10 levels and lower expression of leukocyte surface markers after ITBVI guided fluid therapy compared to HUO guided resuscitation.

In MEDLINE no data exists regarding the type of fluid resuscitation, changes in cytokine profile and expression of leukocyte surface markers in burned patients. Moreover, it has been proven that type of fluid resuscitation can influence the inflammation in cardiac surgery patients and hypovolemic shock can initialize inflammation in burned patients. In our study an increased cardiac index (CI) and $ScvO_2$ could be observed in ITBVI compared to HUO group in the first 24 hours. In our study higher levels of IL-6 were found in the ITBVI group and similarly to Venet and coworkers' findings it was associated with higher CI and $ScvO_2$ which emphasizes the importance of early normalization of oxygen delivery.

Leukocyte cell surface markers play an important role in the initialization of inflammation after burn trauma. We studied the expression and changes of this adhesion molecules. Granulocyte CD11b levels were significantly lower in the ITBVI group from day 4. Granulocyte CD11a/b and monocyte CD11a/b and CD18 levels were significantly lower in the ITBVI group following fluid resuscitation. These results are in accordance with our previous study which showed that granulocytes are less active in the ITBVI group reflected in a lower ROS production. Increased CD11a/b/18 expression as a sign of leukocyte hyper

activation can promote neutrophil adherence to endothelium causing microvascular plugging by leukocytes that, along with edema, can lead to inadequate tissue oxygenisation. When comparing survivors to non-survivors significantly higher levels of CD11a were reported in non-survivors without a significant difference in CD11b and CD18 levels. These data suggest that a less pronounced expression of the above mentioned cell surface markers in the ITBVI compared to HUO group may be beneficial for the burned patient.

CD97 is an inflammatory marker, broadly expressed on hematopoietic cells and is involved in neutrophil migration and leukocyte trafficking. Its role has not been studied in burn injury, but its neutralization increases the resistance to collagen-induced arthritis in mice and its importance has been proven in the cardio-pulmonary-bypass-related inflammatory response. In our patients its expression increased markedly day by day following burn trauma both on granulocytes and monocytes, and even on normally low expressing lymphocytes, reflecting the ongoing inflammatory process. Moreover, CD97 expression was higher on monocytes and lymphocytes in the HUO treated patients indicating the enhanced inflammation in this group.

Carriage of the CD14-159C allele imparted at least a 3.3-fold increased risk of death after burn injury, assessing patients in which deaths were accompanied by severe sepsis. Monocyte CD14 expression was significantly lower in the ITBVI group from day 3 compared to the HUO group. CD14 plays a role in the acute phase response of serum amyloid A and P component in the liver after burn injury. Elevated levels of CD14 were associated with an increased risk of severe sepsis after burn injury. There is growing evidence that after burn injury due to shock, endotoxin can cross the gut wall and enter into the systemic circulation. We postulate that the observed difference in CD14 expression between groups might be a sign of better preserved intestinal circulation suggesting by higher $ScvO_2$ levels and lower MOD score. Decreased endotoxin transmission is suspected as underlying cause of decreased CD14 expression in patients undergoing ITBVI guided fluid therapy.

Burn injury induced oxidative stress gives a good rationale of antioxidant therapy of patients, but only few data exists regarding the effect of the antioxidant therapy in patients suffering from burn injury. In our following study, we found that NAC treatment increased the level of endogenous antioxidants and diminished interleukin production in the acute phase of burn trauma. NAC treatment diminished inflammatory reaction in the acute phase of burn trauma reflected in lessened leukocyte cell surface marker expression. The need for inotropic and vasopressor drug administration significantly decreased in NAC treated patients.

Oxidative stress can be evaluated by either measuring the end products of lipid peroxidation, or the antioxidant capacity and the activity of antioxidant enzymes. NAC is widely used in clinical practice. NAC is not an endogenous antioxidant, but, its use is based on a convincing rationale. In animal model administration of NAC increased GSH and decreased MDA levels in the lung 1 hours and 1 day post burn injury. Treatment of rats with NAC significantly elevated the GSH, while decreasing MDA level and MPO activity after burn injury. In humans NAC administration increased serum GSH in patients undergoing IL-2 induced lymphokine-activated killer cell treatment. GSH were significantly higher in our NAC treated patients from day 2, but in contrast to earlier results NAC treatment did not alter plasma MDA level. The close correlation between stimulated ROS production in whole blood and inflammation can explain the effect of NAC on cytokine production. The anti-inflammatory effect of NAC can be based on regulatory effect of ROS on translocation of transcription factor NF-κB to cell nuclei. Moreover, IL-8 production is regulated by mitogen-activated protein kinases, but ROS can alter IL-8 production too.

Toumpanikis and associates found that antioxidant supplementation with NAC decreased interleukin production of monocytes at rest and exercise in humans. Radomska-

Lesniewska and associates concluded that NAC is an effective inhibitor of TNFα, IL-1β and IL-8 release in endothelial and epithelial cells. Our data are in concordance with these results showing a less pronounced cytokine production in NAC treated patients. The data of recent study support the results of our earlier work in which a diminished inflammatory reaction reflected in lessened leukocyte cell surface marker expression was observed after NAC treatment.

Among β2 integrins granulocyte CD11a levels were significantly lower in the NAC group from day 4 following injury, similarly to CD18. A less pronounced expression of the cell surface markers in the NAC group may be beneficial. CD49d, similarly to CD11 and CD18 is an adhesion molecule and plays an important role in the tight cell to cell (leukocyte-endothelium) interaction. CD49d mediates a CD18-independent neutrophil accumulation. It has been shown to be involved in lymphocyte, eosinophil, and monocyte adhesion and emigration. Its role in burn has not been investigated yet. Normally, CD49d is poorly expressed on granulocytes. In our study, granulocyte CD49d expression was significantly higher in standard group on day 2 only, whereas the expression of lymphocyte CD49d was less pronounced from day 3, and expression of monocyte CD49d from day 4 in NAC group, suggesting the beneficial effect of NAC treatment.

CD97 is a member of the G protein-coupled receptor. In our patients, its expression increased markedly day by day on the granulocytes and monocytes in the standard group. Its levels were significantly lower in NAC group from days 2 and 3. No significant differences could be observed on normally low expressing lymphocytes.

In our study, the major lipopolysaccharide binding receptor CD14 levels showed a decreasing tendency during the study period in both groups, without any significant intergroup differences, indicating a similar LPS exposition.

In a porcine model NAC did not influence the early post-traumatic organ injury, and initiation of inflammatory responses significantly, or endotoxin tolerance. In vitro, NAC significantly reduced pro-inflammatory cytokine release in normal blood only. In human studies NAC treatment did not influence the survival. In this study a beneficial effect of NAC treatment could not be proven on mortality rate. The less pronounced inflammation - reflected in lower interleukin levels in NAC group - can explain the lower inotropic and vasopressor drug requirements of NAC treated patients. Moreover, the late onset of the effect of NAC treatment (after edema formation was complete) can explain the lack of difference in fluid requirements between groups.

The study is underpowered regarding clinical outcome parameters, except for the inotropic and vasopressor drug requirements. The low number of patients may be the underlying cause of absent statistical differences between groups, although mortality rate was higher in the standard group. The less pronounced SIRS was reflected in lower CD marker expression.

7. Novel findings

7.1. Time course of pro- and anti-inflammatory cytokine and HMGB1 levels in patients with burns

- Our results confirmed that an overwhelming anti-inflammatory response after burn reflected in marked elevation of IL-10 levels is associated with more frequent occurrence of sepsis and higher mortality rate. Higher levels of IL-10 on admission showed a good predictive value.
- The results demonstrate a very early increase in plasma HMGB1 within 5 h of burn injury in humans. HMGB1 levels were significantly higher in septic as well in nonsurviving patients and have good predictive values regarding both sepsis and mortality.

7.2. Effects of fluid resuscitation methods on the pro- and anti-inflammatory cytokines and expression of adhesion molecules after burn injury

- This study demonstrates significantly higher IL-6, significantly lower IL-10 levels and lower expression of leukocyte surface markers after ITBVI guided fluid therapy compared to HUO guided resuscitation.
- Our data suggest that a less pronounced expression of cell surface markers in the ITBVI compared to HUO group may be beneficial for the burned patient.
- The results of our study together with our previous examinations suggest that ITBVI directed shock treatment comparing to HUO guided fluid resuscitation is associated with earlier normalization of oxygen supply and demand ratio and less pronounced shift of cytokines towards anti-inflammatory imbalance and expression of leukocyte surface markers in burned patients.

7.3. The effect of NAC treatment on the oxidative stress, expression of leukocyte surface markers and pro- and anti-inflammatory cytokines after burn injury

- We have found that NAC treatment increased the level of endogenous antioxidants and diminished interleukin production in the acute phase of burn trauma.
- In this study, we found that NAC treatment diminished inflammatory reaction in the acute phase of burn trauma reflected in lessened leukocyte cell surface marker expression.
- The need for inotropic and vasopressor drug administration significantly decreased in NAC treated patients.

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9. List of publications

This thesis is based on the following publications

1. Csontos C, Rezman B, **Foldi V**, Bogar L, Bognar Z, Drenkovics L, Röth E, Weber G, Lantos J. Effect of N-acetylcysteine treatment on the expression of leukocyte surface markers after burn injury. Burns 2011; 37: 453-64. **IF: 1.718**
2. **Foldi V**, Lantos J, Bogar L, Roth E, Weber G, Csontos C. Effects of fluid resuscitation methods on the pro- and anti-inflammatory cytokines and expression of adhesion molecules after burn injury. J Burn Care Res 2010; 31: 480-91. **No. of citation: 1; IF: 1.563**
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Cumulative IF of publications related to the thesis: 9.819

Other publications

1. Bognar Z, **Foldi V**, Rezman B, Bogar L, Csontos C. Extravascular lung water index as a sign of developing sepsis in burns. Burns 2010; 3: 1263-70. **No. of citation: 1; IF: 1.718**
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No. of citation: 3; IF: 1.953

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This thesis is based on the following abstracts appeared in journals

1. **Földi V**, Lantos J, Bogár L, Rőth E, Wéber G, Rézmán B, Drenkovics L, Csontos C. A fehérvérsejtek sejtfelszíni marker expressziójának változása égési sérültekben N-acetilcisztein kezelés hatására. Aneszteziológia és intenzív terápia 2010; 40(S1): 12.
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12. **V. Foldi**, C. Csontos, L. Bogar, E. Roth, J. Lantos. The kinetics and prognostic role of IL-10 in patients with burn injury. Abstracts from the Hungarian Society of Anesthesiology and Intensive Therapy, 36th Annual Conference. Journal of Critical Care 2009; 24: 146–148. **IF: 2.127**
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Cumulative IF of abstracts related to the thesis: 38.138

Cumulative IF of publications and abstracts related to the thesis: 47.957