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

ABA - American Burn Association
ADH - antidiuretic hormone
ANP - atrial natriuretic peptide
APC - antigen presenting cell
APACHE - acute physiologic assessment and chronic health evaluation
ARDS - adult respiratory distress syndrome
AUC - area under curve
ATP – adenosine triphosphate
BBS - burnt body surface
BSA - body surface area
CABG - coronary artery bypass graft
CAMs - cell adhesion molecules
CARS - compensatory anti-inflammatory response syndrome
CAT - catalase
CI - cardiac index
CI_v - confidence interval
CL - chemiluminescense
CO - cardiac output
CD - cluster of designation/differentiation
CVC - central venous catheter
CVP - central venous pressure
DNA - deoxyribonucleic acid
DMEM - Dulbecco's modified Eagle's medium
DO_2 - oxygen delivery
DTNB - 5,5'-dithiobis(2-nitrobenzoic acid)
ECM - extracellular matrix
EDTA - ethylene diamine tetraacetic acid
ELISA - enzyme-linked immunosorbent assay
ERK - extracellular signal-regulated kinase
GR/GPX - glutathione reductase/peroxidase
GPX - glutathione peroxidase
GSH - reduced gluthation
H_2O_2 - hydrogen peroxide
HMGB1 - high mobility group box protein-1
HUO - hourly urine output
ICAM-1 - inter-cellular adhesion molecule-1
ICU - intensive care unit
IG - immunoglobulin
IL - interleukin
ITBVI - intrathoracic blood volume index
IU - international unit
LPS - lipopolysaccharide
LR - lactated Ringer solution
IQR - interquartile range
LIS - lung injury score
MAP - mean arterial pressure
MAPK - mitogen activated protein kinase
MDA - malondialdehyde
MOD - multiple organ dysfunction
MODS - multiple organ dysfunction score
MOF - multiple organ failure
MPO - myeloperoxidase
NAC - N-acetylcysteine

NADPH - nicotinamide adenine dinucleotide phosphate-oxidase

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NO - nitrogen monoxide

NOS - nitrogen monoxide synthetase enzyme

NS - non-significant

O_2 - oxygen

O_2^- - superoxide radical

$OH^{\cdot}$ - hydroxyl radical

OR - odds ratio

PAI-1 - plasminogen activator inhibitor 1

PBS - phosphate buffer solution

PF - Parkland formula

PMA - phorbol-12 myristate-13 acetate

PMNL - polymorphonuclear leukocytes

PSH - protein sulfhydryl groups

RAGE - receptor for advanced glycation end products

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

TBARS - thiobarbituric acid reactive substances

TBSA - total burnt surface area

TCA - trichlore acetic acid

TH1 - T-helper type 1 cell

TLR2/4 - toll-like receptor 2 and 4

TNFα - tumor necrosis factor α

tPA - tissue plasminogen activator

Tx-A_2 - thromboxane-A_2

UV - ultraviolet

VCAM-1 - vascular-cell adhesion molecule 1

VLA - very late antigen

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. It is one of the most common injuries - mainly in developing countries. 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[1]. Immunodeficiency can develop because of the reduced immunoglobulin synthesis (down-regulation), with consequence of an increased acquisition of infection[2,3]. 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[4]. 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[5]. 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.

2.1. Pathophysiology of burn edema

2.1.1. The mechanism of burn edema formation

Ernest Starling published his theory in 1896, where he described that the capillary hydrostatic pressure, forcing fluid out of the capillary into interstitium, was counterbalanced by the colloid osmotic pressure, produced by plasma proteins, which holds fluid in the capillary lumen.

Edema formation is provoked by physical forces and changes in the structure of capillaries and interstitium. Hydrostatic and oncotic pressure both in the capillaries and interstitium are the main regulative factors. The interference of these pressures and forces are demonstrated in Figure 1.

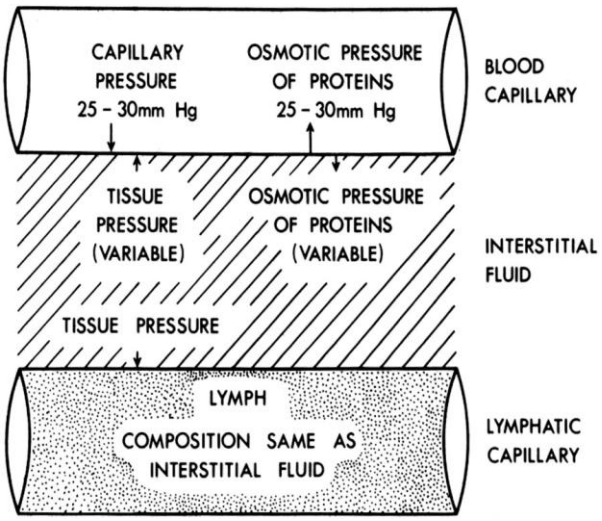


Figure 1.: Physical factors controlling edema. Hydrostatic and osmotic forces are described. In addition, the interstitium itself is shown, with its own physical properties, controlling fluid accumulation. Intact lymphatics are important for fluid clearance[6]

Ideally, there is equilibrium between the counterbalanced forces; the fluid flux into the interstitium is minimal, which is transported by the lymphatic network. If equilibrium shifts, fluid accumulates in the interstitium and edema occurs.

2.1.2. Factors affecting edema formation

Starling's equation is applicable in burn too, but the certain factors among them dominate more. Intersticial compliance; the evidence that both the collagen and hyaluronic acid components of matrix are fragmented would lead to a dramatic increase in fluid accumulation because these are the tethering molecules in the interstitium. In addition, much of fluid accumulates in the tissue plane between dermis and subcutaneous fluid after full-

thickness burns. The compliance of this space, like any space composed of loose connective tissue, should increase rapidly with fluid accumulation. The gain of interstitial compliance is crucial in the mechanism of burn edema formation[6].

Mainly hyaluronic acid molecules compose the interstitial oncotic pressure. Destruction of the collagen and hyaluronate spring and the other matrix components in the dermis would greatly diminishes any restraining force capable of limiting further matrix swelling[6].

2.1.3. Edema formation in partial-thickness and deep burn injury

The kinetic of edema formation and the driving force substantially differ in partial-thickness and deep burn injury. Circulation and lymphatic network of the skin is intact in *partial-thickness burn*; the main driving force of edema formation is the increase in the capillary permeability. The improved lymphatic flow cannot keep pace with the degree of extravasation. In case of partial-thickness burn edema formation reaches its maximum 12-18 hours after injury; usually 94% of edema still persists 6 hours after trauma. After 24 hours resorption begins and due to the intact lymphatic network resorpts in about 4 day[7]. *Deep burn injury* damages both the circulation and the lymphatic network of the skin. Hyaluronic acid fragments and therefore the increased interstitial osmotic pressure constitute the driving force of edema formation. Edema takes up position in the deeper layers of the skin, between epidermis and dermis. The kinetic of edema formation is much slower, it reaches the maximum 18 hours after injury or later. Although resoprtion starts 24 hours after injury, 25% of the total edema is still there 7 days afterward burn trauma[8].

2.2. Fluid resuscitation

In burn disease therapy adequate fluid resuscitation plays a very important role[9]. The aims are to substitute circulating volume, maintain circulation and prevent or treat hypovolaemic shock. In spite of that infusions may increase the amount of edema; fluid resuscitation is our most important assistance to maintain the circulation[10]. The objective of adequate fluid resuscitation is to maintain oxygen delivery without much increase in the interstitial edema formation[11].

Empirical fluid resuscitation schemata often underestimate the fluid need of burn patients, what results in inadequate fluid resuscitation[12,13,14,15]. The advantage of *non-invasive endpoints* is the good applicability, no need for invasive intervention, but their information content is for these reasons limited[16,17]. The use of *invasive endpoints* by

transcardiopulmonary thermodilution techniques allow a more adequate treatment of hypovolemia[18,19,20,21,22,23]. The adequate endpoint is under debate. It follows that the suitable and ultimate fluid resuscitation method is still missing.

Another possibilitiy is to reduce fluid requirement of burned patients via influence on the underlying pathophysiological processes of edema formation. The prevention or lessens in burn edema formation could decrease the fluid loss to the interstitium, hypovolaemia and the fluid need of burned patients will decrease. To understand these target parameters we should monitor the pathopysiological changes in burn disease.

2.3. Pathophysiology of burn trauma induced inflammation

2.3.1. Free radicals

2.3.1.1. Free radical production

Free radical production has a multivarious way. Some of these reactions are presented normally in the organism, while others activate only in pathological circumstances.

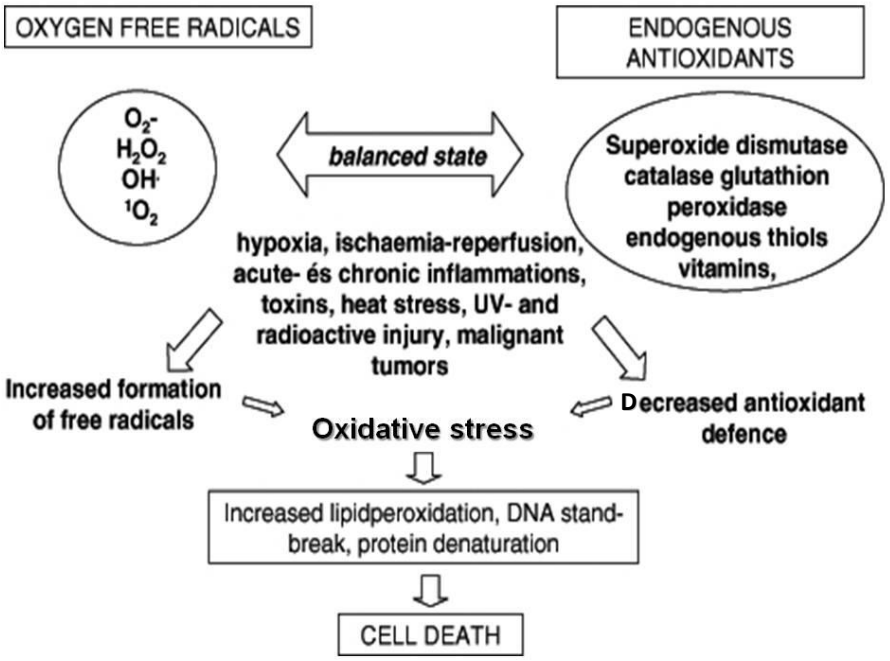
One of the sources of free radicals is the mitochondrial electron transport chain. The cytochrome oxidase enzyme complex (Complex IV) transforms O_2 to water in a reductive reaction. However this reaction is not accomplished; in the course of O_2 reduction superoxide radical (O_2^-) occurs which is transformed to hydrogen peroxide (H_2O_2) in spontaneous or enzymatic dismutation. Mitochondrial electron transport chain is the main source of H_2O_2 in cells[24]. Catalytic decomposition of H_2O_2 produces hydroxyl radical (OH^-). Free radical production from fatty acids through peroxisomal β oxidation is a potential pathway. The by-product is H_2O_2 in this reaction too. Cytochrome p-450 enzyme is able to reduce O_2 to O_2^-. The normal rate of this reaction is unknown, but in pathological circumstances these reactions could be important in the development of oxidative stress[25,26].

Oxidative burst is the overproduction of reactive oxygen species (ROS), which is needed to protect the body against external threats, but it could also lead to severe tissue damages. Leukocytes start to produce ROS (O_2^-, H_2O_2, $OH^\cdot$) against external insults[27,28,29]. Leukocytes are able to produce hypochlorous acid from free radicals and utilize it to produce $OH^\cdot$. NADPH oxidase produces NADP + $2H^+$, the by-product in this reaction is O_2^-. In spontaneous or enzymatic route H_2O_2 origins from this O_2^-. Myeloperoxidase (MPO) enzyme produces with Cl^- hypochlorous acid from this H_2O_2 in cells. Hypochlorous acid reacts with superoxide anion, which reacts with Fe^{2+} and $OH^\cdot$ arise. Nitrogen monoxide (NO) synthetase enzyme (NOS) oxidates L-arginine to L-citrulline; NO releases. Three isoforms of this enzyme are known. Neuronal and endothelial isoforms play a part in the physiological NO

production. Inducible NO synthetase generates high amount of NO in the course of inflammatory reactions which could produce with O_2^- extremely aggressive peroxynitrite. Peroxynitrite plays a part in the activation of cyclooxygenase and it works as a signalling molecule, depolarisates the mitochondrial membrane and disconnects the oxidative phosphorilation and the ATP synthesis[30].

2.3.1.2. The role of free radicals in different clinical aspects

The pathological role of free radical reactions have been proven in several conditions (hypoxia, ischaemia-reperfusion syndromes, acute- and chronic inflammation, poisonings, burns, uv- and radioactive radiation injuries, tumors). During so-termed pathological free radical reactions the number of reactive radicals multiplies; the capacity of endogenous antioxidant neutralization system becomes insufficient[31]. This condition is often augmented by the damage of the proteins with antioxidant capacity (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX)) which leads to the lesion of macromolecules. Increased free radical production and insufficient antioxidant capacity leads to oxidative stress, which severely impairs the cell function (Figure 2.). Oxygen-centred free radicals have causal role in the pathogenesis of several diseases and in others they are responsible for the progression of the already envolved state. The most important documented role of free radicals is in the development of ischemic-reperfusion injuries, cardiovascular diseases, diabetes, haemorrhagic shock, atherosclerosis and hypertension[32,33,34,35,36,37]. The pathological role of free radicals has been proven in alcoholic liver disease, chronic hepatitis C infection[38], or rather in pulmonary embolism[39]. Surgical trauma could also evoke oxidative stress reaction[40]. Oxidative stress has a pathophysiological role in the development of polytrauma related molecular processes (Figure 2.)[41], as well as in burn disease. Burn injury induces sustained oxidative stress response in humans.



H_2O_2: Hydrogen peroxide; O_2^-: Superoxide anion; OH•: Hydroxyl radical; UV: Ultraviolet

Figure 2.: Oxidative balance of the cell: development of oxidative stress[42]

2.3.1.3. The role of free radicals in burn disease and burn edema formation

The ROS, released during burn disease, attack the unsaturated fatty acid rich cell membrane. Lipid peroxidation is one of the most dangerous pathological reactions during burn injury[43,44]. Lipid peroxidation triggers changes in the cell membrane, therefore markedly damages the function of cell membrane related proteins[45]. There is a close correlation between the severality of lipid peroxidation, burn related organ failure and burn shock[46,47]. Burn injury causes intravascular neutrophyl granulocyte activation, which leads to increased ROS production. ROS oxidate the phospholipid membranes of cells. Lipid peroxidation end-products manifest in the burn affected tissues, edema and lympha; this shows the role of this oxidative pathomechanism. Free radical accumulation in the early phase of burn disease could be observed abreast with edema formation[48,49].

Free radicals take a part in burn edema formation in two different ways. One of the above described mechanisms damages the cell membrane, capillary permeability increases, albumin crosses over without resistance in the first 24 hours after injury. On the other hand free radicals damage hyaluronic acid, collagen structures, and other matrix elements[50,51]. These procedures contribute in burn edema formation due to increased interstitial compliance and osmotic active products.

Our earlier study suggested that total burnt surface area (TBSA) over 15% doesn't show a correlation with burn injury induced oxidative stress. Our other study proved that fluid resuscitation regimes have different impacts on the prooxidant status, mainly on granulocyte function, but not on the changes in endogenous antioxidants in burned patients[52]. This observation indicated an improved preservation of organ functions and elevated oxygen saturation of the central venous hemoglobin ($ScvO_2$) using intrathoracic blood volume index (ITBVI) guided fluid resuscitation. A moderately diminished pro-oxidative capacity was associated with ITBVI guided fluid administration in severely burned patients[52].

2.3.1.4. The role of N-acetylcysteine (NAC) and other antioxidant substances in the treatment of burn injury induced oxidative stress

The oxidative stress plays an important role in the edema formation proven by experimental research, where allopurinol[49,53,54], or other antioxidant pre-treatment significantly decreased edema formation. For antioxidant therapy desferroxamine (15 mg kg^{-1} day^{-1}), allopurinol (50 mg kg^{-1} day^{-1}), NAC (1 mg kg^{-1} day^{-1}) and SOD (10000 U kg^{-1} 6 h^{-1}) were used. Out of these compounds NAC is the most studied, which is the known precursor of glutathione. Administration of vitamin C 6 hours after burn injury in 60 mg kg^{-1} h^{-1} dose significantly decreased burn edema formation[55]. Vitamin C decreased significantly the downdraft of the interstitium and for this the edema formation in deep burn injury. Others studies[56,57] reported the positive effect of antioxidants on capillary permeability. These results are based on animal experiments, only few adequate human clinical studies exist[58].

Antioxidant system plays an important role in protection against the harmful effects of ROS. SOD, CAT and glutathione reductase/peroxidase (GR/GPX) are the enzymatic parts of this antioxidant system. SOD converts O_2^- to less harmful H_2O_2; CAT plays a basic role in the H_2O_2 and organic peroxide neutralization. SOD, CAT and GR/GPX system protects from the damaging effects of free radicals; SOD, CAT and GR/GPX activity increases in the early phase of free radical production and lipid peroxidation[59].

The main part of non-enzymatic protection system is represented by the plasma sulphydryl groups (PSH) which play an important role in the binding of ROS. Normally the reduced and oxidized glutathione ratio is high in cells; glutathione reductase generates high amount of reduced glutathione which is able to bind the ROS[60,61]. Tissue hypoxia, following burn trauma, leads to intracellular reduced gluthation (GSH) depletion, may caused by the high GSH utilization through free radical activity[62,63]. Its effect is well studied in animal models. The study of Konukoglu and associates showed that thermal injury is associated with

increased pulmonary lipid peroxidation[64]. NAC treatment preserved the organ function and GSH level was higher in NAC treated rats[64]. Use of NAC resulted in the significant improvement of burn induced immunosuppression, as reflected by contact hypersensitivity response in rats. The early intervention of antioxidant therapy was able to significantly restore cell-mediated immunity[65]. In a rat burn model, pre- and post-burn administration of NAC prevented burn-induced bacterial translocation, reflected in decreased incidence of isolating bacteria in mesenteric lymphnodes, spleen, and liver specimens. Treatment of rats with NAC significantly elevated the reduced GSH levels, while decreased malondialdehyde (MDA) levels and MPO activity[66].

SOD activity usually decreases after burn trauma due to the activated SOD deterioration[67,68]. Experimental and clinical data suggests that exogenous SOD therapy was effective fencing off the ROS caused damages[43,44,69,70]. Administration of SOD derivates in 1 mg kg^{-1} dose moderately decreased the concentration of lipid peroxidation end-products in serum, lung and kidney in early stage of burn disease in rats[44]. Of course, other antioxidant compounds came into consideration of researchers and clinicians. Use of antioxidants decreased burn edema formation and tissue damages caused by ROS in animals[71,72]. Intravenously administered NAC decreased the ischemia-reperfusion damages through the ROS-binding ability[73], moreover NAC decreased the level of inflammatory citokines, the expression of adhesion molecules and inhibited the activity of NF-κB[74,75]. Animal experiments proved, that NAC supplementation (150 mg kg^{-1}) 15 minutes before and 2 hours after burn injury significantly increased the GSH level in the circulation, and significantly decreased serum MDA level and MPO activation and minimized bacterial translocation[66].

Human studies have found decreased serum vitamin A and C, as well as selenium level, and increased lipid peroxidant end-products after burn injury[76]. The antioxidant therapy (vitamin C, NAC) reduces the tissue lesions and increases the protective ability of the body against oxidative damages[77,78]. According to our knowledge the administration of vitamin C (0.5-14.2 mg kg^{-1} day^{-1}), as well as vitamin E (1200 IU day^{-1}) are useful supplementation in the treatment of burn patients. Tanaka et al.[79] administered higher doses of vitamin C in animal experiments. After a 66 mg kg^{-1} bolus, 33 mg kg^{-1} h^{-1} vitamin C infusion was given, which decreased burn edema markedly. Vitamin C doesn't have an own antioxidant effect, it is important in the rereduction of GPX, because it is the co-enzyme of GPX reductase.

Although a lot of studies have proven that antioxidants play an important role in the treatment of burn injury and burn related oxidative stress, there is no consensus which antioxidants and which dose are needed in the clinical practice.

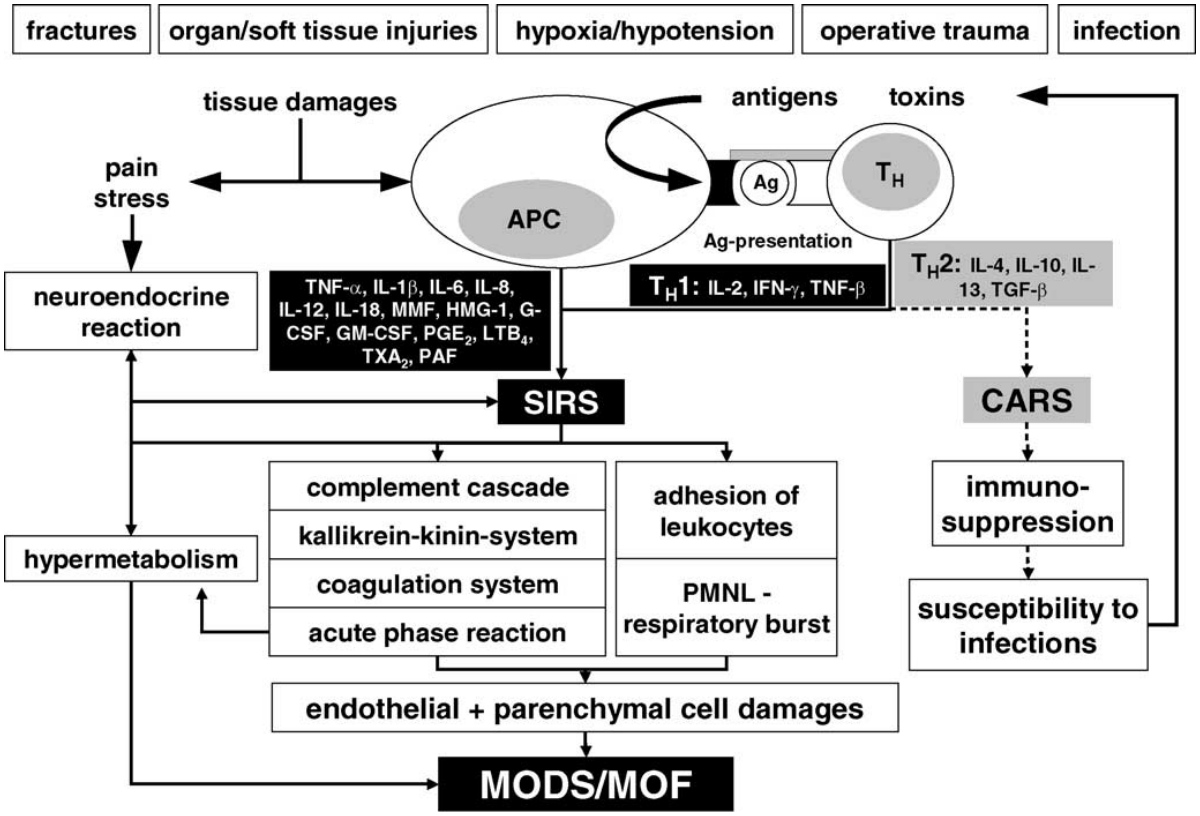
2.3.2. Cytokines and adhesion molecules

Cytokines play an important role in the pathophysiological processes after burns[80,81,82]. Leukocyte surface markers also play an important role in the initialization of inflammation after burn trauma. CD11a, CD11b, CD18, CD49d are adhesion molecules contribute to tight cell to cell (leukocyte-endothelium) connection, leukocyte activation[83,84,85,86,87], activation of cell mediated immunity[88,89,90,91] and involved in neutrophyl migration[92] and leukocyte trafficking[93].

2.3.2.1. Cytokines

Cytokines are small cell-signaling protein molecules that are secreted by the glial cells of the nervous system and by numerous cells of the immune system and are a category of signaling molecules used extensively in intercellular communication. Cytokines can be classified as proteins, peptides, or glycoproteins. The term "cytokine" has been used to refer to the immunomodulating agents, such as interleukins and interferons. Virtually all nucleated cells, but especially endo/epithelial cells and resident macrophages (many near the interface with the external environment) are potent producers of IL-1, IL-6, and TNFα (Figure 3.).

Although the quality of burn wound- and intensive care has made a considerable progression sepsis and complications of inhalation injury remained the leading cause of morbidity and mortality after burn. The elevated level of circulating cytokines produced by T and B lymphocytes, monocytes, macrophages and keratinocytes has already been observed suggesting their important role in the pathophysiological responses following burn injury[80,81,82,94].



APC: antigen presenting cells; TH: T-helper cells (lymphocytes); SIRS: systemic inflammatory response syndrome; CARS: compensatory anti-inflammatory response syndrome; PMNL: polymorphonuclear leukocytes; MODS: multiple organ dysfunction syndrome; MOF: multiple organ failure

Figure 3.: Host defence response after trauma[41].

TNFα, IL-6 and IL-8 play important role in the acute phase inflammatory response to trauma. TNFα is a potent mediator of the inflammation and induces a cascade of secondary cytokines (IL-6, IL-1β)[95,96,97]. IL-6 takes part in the development of inflammatory response, and has prognostic significance, as it's level shows good correlation with the severity of burns and survival[98,99]. IL-6 is a key cytokine in B-lymphocyte regulation and acute phase protein induction[100] and its serum level correlates with the extent of burn injury[101] however, it does not produce signs of septic shock when administered to animals in contrast to TNFα and IL-8. IL-1β is not only a potent pro-inflammatory cytokine but it up-regulates the expression of 26s proteasome in rats and causes a hypermetabolic state[102], although it has been suggested that serum levels of IL-1β are not increased after burn[103]. Higher levels of IL-1β were found in the central nervous system, in the lung and liver tissues of rats after thermal injury[104]. IL-8 recruits inflammatory cells to sites of injury. Serum levels of IL-8 increases rapidly after injury and this increase correlates well with the development of adult respiratory distress syndrome (ARDS) and mortality from multiple organ failure (MOF)[105]. IL-10 plays an

important role in the inhibition of inflammatory responses[106] and in trauma patients higher levels of IL-10 appeared to correlate with the development of sepsis[107]. Early antagonism of IL-10 in mice can improve the chances of survival[108]. The blocking of TNFα and IL-10 in animal models could decrease the organ damage and increase survival[109]. An elevated level of IL-12 has been found in children on the second week after injury[80]. It is produced primarily by antigen-producing cells and plays a primary role in the induction of cell-mediated immunity. IL-10 can suppress IL-12 formation. IL-10 knocked out mice showed a higher IL-12 response to injury with an earlier death compared with wild type mice[105]. Elevated levels of IL-6, IL-8 and IL-10 have been found in burned children on the third day after injury[80]. According to the literature, a shift can be observed towards anti-inflammatory cytokine production after burn trauma[81]. This shift marked with an elevated level of IL-10 leads to decreased resistance to infections[82,110,111].

Although, the role of the different cytokines has been well studied, the dynamism and the prognostic role of the elevated cytokines are not fully explained in humans regarding the development of bacterial sepsis.

2.3.2.2. Adhesion molecules

Adhesion molecules are proteins located on the cell surface involved in the binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion. These proteins are typically transmembrane receptors and are composed of three domains: an intracellular domain that interacts with the cytoskeleton, a transmembrane domain, and an extracellular domain that interacts either with other cell adhesion molecules (CAMs) of the same kind (homophilic binding) or with other CAMs or the extracellular matrix (heterophilic binding). They play vital roles in numerous cellular processes. Some of these include: cell growth, differentiation, embryogenesis, immune cell transmigration and response, and cancer metastasis. Adhesion molecules are also capable of transmitting information from the extracellular matrix to the cell. There are four major families of cell adhesion molecules. These are the immunoglobulin (Ig) superfamily, integrins, cadherins, and selectins.

Leukocyte cell surface markers play an important role in the initialization of inflammation after burn trauma. CD11a, CD11b, CD18 and CD49d (cluster of designation/differentiation - CD) are adhesion molecules, and contribute to tight cell to cell (leukocyte-endothelium) connection and leukocyte activation[83,84,85,86,87]. The β2 integrins (CD11a, CD11b and CD18) are expressed on all leukocytes, while VLA-4 (CD49d) as member of the β1 integrin subfamily is involved only in lymphocyte and monocyte

adhesion[112]. CD14 is present exclusively on monocytes. It is the major lipopolysaccharide (LPS) receptor and plays an important role in the activation of cell mediated immunity[88,89,90,91]. CD97, with its three different isoforms[89], is a member of the adhesion molecule family of G protein-coupled receptors[92], it is heavily expressed on hematopoietic cells and it is involved in neutrophil migration[92] and it also plays a role in leukocyte trafficking[93].

2.3.2.3. High-mobility group box protein 1

High-mobility group box protein 1 (HMGB1) is a cellular protein discovered 30 years ago as a nuclear binding protein[113]. HMGB1 is active in DNA recombination, repair, replication and gene transcription, facilitated by internal repeats of positively charged domains of the N terminus (HMG boxes)[114]. HMGB1 was also identified as a late mediator of systemic inflammation. HMGB1 is present in the nucleus of mammalian cells[115,116]. The structure of the HMG-box domain consists of three helices in an irregular array. Proinflammatory activation signals caused by infections induce an active release of HMGB1 from activated monocytes and macrophages[117,118] (Figure 4.)[119] and it is also released passively by necrotic and damaged cells[120]. Extracellular HMGB1 is a danger signal to responsive cells and amplifies the signal by increasing production and secretion of other proinflammatory mediators (TNFα, IL-1β, IL-6, IL-8, etc) and finally induces[119,121,122] inflammation (Figure 4.)[119].

HMGB1 stimulates expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and receptor for advanced glycation end products (RAGE), as well as secretion of TNFα, IL-8, monocyte chemotactic protein-1, plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator[119]. These findings link HMGB1 to regulation of the coagulation system and underscore its role as a pro-inflammatory mediator[119,123].

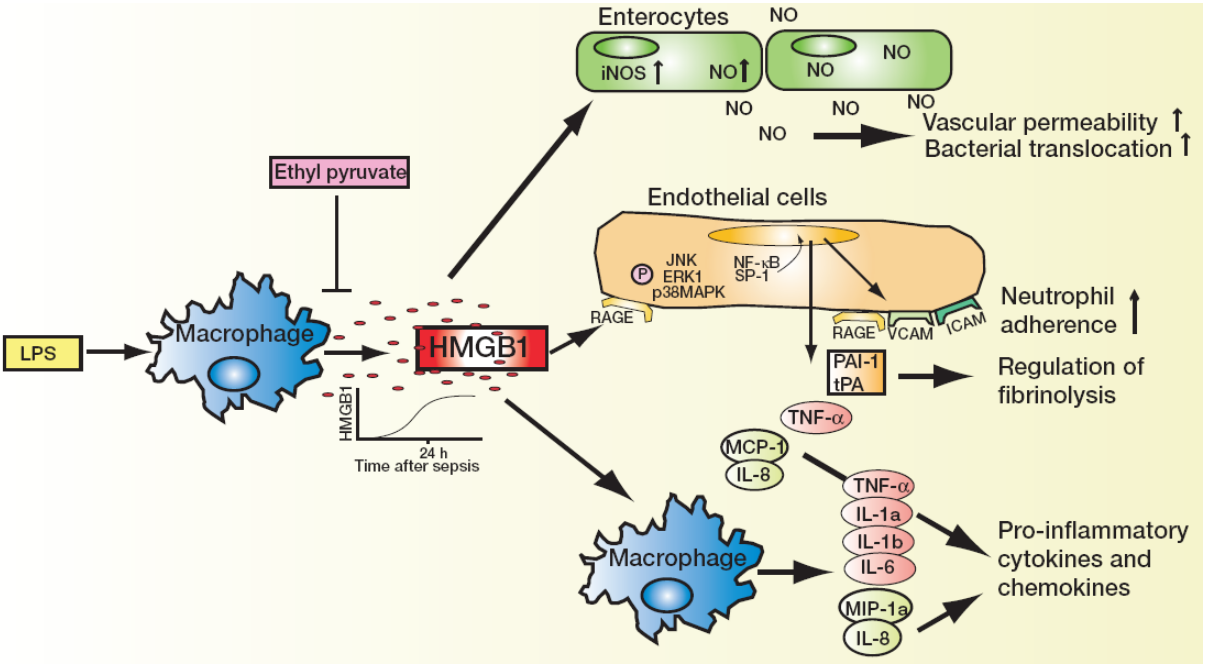


Figure 4.: Contributions of HMGB1 to inflammation. (See text) [119].

In apoptotic cells, HMGB1 is firmly bound to hypoacetylated chromatin and does not transmit the inflammation signal[124]. Serum HMGB1 level is low in healthy humans and significantly elevated in different pathological states. One study in patients with sepsis showed higher plasma concentration in non-survivors than survivors[125], but the results were contradictory in another study[126]. Burn injury is associated with severe hypovolemia caused by excessive edema formation.

HMGB1 may also be involved in the pathogenesis of hemorrhagic shock[127]. Plasma HMGB1 is significantly increased within 1 h of mechanical trauma in humans, with marked elevations occurring from 2 to 6 h postinjury[128]. Several experimental data suggest that burn injury induces significant elevation of plasma HMGB1 concentration[129,130], but there are only few data available about the changes in plasma HMGB1 concentration in humans[131]. The time course of plasma HMGB1 in the immediate postinjury period and their prognostic value in patients with severe burn injury has not yet been clarified.

2.3.3. The effect of NAC treatment on the inflammation

In a lung ischemic-reperfusion model administration of NAC prior to or shortly after circulatory arrest resulted in a marked reduction of inflammation during the warm ischemic phase[132]. Toll-like receptor 2 and 4 (TLR2/4) may play an important role in ischemia-reperfusion injury. NAC inhibited the activation of TLR2/4 and the induction of TNFα resulting from ischemia-reperfusion injury via modulating the redox state in mice liver and lung tissue[133]. In contrast to these results, Gundersen and associates found in a pig polytrauma model that adding NAC to the immediate resuscitation fluid did not influence the early post-traumatic organ injury and initiation of inflammatory responses or endotoxin tolerance significantly. In vitro, NAC significantly reduced proinflammatory cytokine release, but only in normal blood[134].

In experiments performed on human umbilical vein, NAC treatment could inhibit the vascular endothelial cadherin associated increase in vascular permeability[135]. In chronic obstructive pulmonary disease, the initial step in the inflammatory process is the overexpression of adhesion molecules, which leads to excessive transmigration of neutrophils. NAC administration inhibited the TNFα/IL-1β-stimulated ICAM-1 expression[136]. High-dose NAC is a well-tolerated and safe medication for a prolonged therapy of patients with cystic fibrosis[137]. Comparing patients treated with NAC to patients receiving standard care suffering from inhalation injury, the NAC treatment significantly improved the lung injury score (LIS), lung resistance and hypoxia scores, moreover, a statistically significant survival benefit could be observed that was most pronounced in patients with APACHE-III scores>35[138]. In the study of Karen and associates, NAC did not prevent postoperative renal dysfunction, interventions, complications, or mortality in high-risk patients undergoing CABG surgery[139].

2.3.4. Sepsis in burn injury

Severe burn injury induces a temporal shift in immune reactivity that can result in septic syndrome or even death. Despite significant advances in intensive care technologies and high enthusiasm in the development of new antibiotics, severe sepsis after burns still claims for 40–50% mortality in many countries[140]. The inflammatory response is triggered immediately after thermal injury and persists for almost 5 weeks postburn[141]. The current definitions of sepsis (fever, tachycardia, tachypnea, and leukocytosis) are less applicable in burns, because these signs still exist in this patients[142]. Burn patients, by definition, already have SIRS. In 2007 the American Burns Association Consensus Conference[142] defined the infection and sepsis criteria in burned patients.

Sepsis criteria in burn injury[141]: Signs of developing sepsis are the following:

Temperature: >39°C or <36.5°C;

Progressive *tachycardia*: heart rate >110 bpm (in adults);

Progressive *tachypnea*: respiratory rate >25 min^{-1} not ventillated, in ventilated patients minute ventilation >12 l min^{-1};

Thrombocytopenia (will not apply until 3 days after initial resuscitation): platelet count <100 G l^{-1} (in adults);

Hyperglycaemia (in the absence pre-existing diabetes mellitus): untreated plasma glucose >11 mmol l^{-1} or intravenous insulin requirement >7 U h^{-1} or 25% increase in insulin requirement over 24 h;

Inability to continue *enteral feedings* >24 h: abdominal distension, enteral feeding intolerance (two times residual feeding rate in adults), uncontrollable diarrhea (>2500 ml d^{-1} for adults).

In addition, for sepsis diagnosis the *identification of bacterial focus* was required: culture positive infection, pathologic tissue sourced identified or clinical response to antimicrobials.

3. The aim of our studies

Burn trauma induces severe oxidative stress and leukocyte activation. Oxidative stress and SIRS play an important role in edema formation, causing severe hypovolemia following burns[6]. A more adequate fluid resuscitation regime guided by 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 exist 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 is 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 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 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, and they were informed clearly about the details of the study and blood sampling. 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 body surface, 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% TBSA 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 treatment. Patients were treated in a uniform way and practice patterns were not changed during the study period. If inhalation injury was suspected (facial burn, soot in the throat, chest X-ray) bronchoscopy was carried out for verification. Excision and grafting were started within 72 hours. 20-30% of burnt surface 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 in order to avoid complications due to coagulopathy.

4.2. Methods

4.2.1. Fluid resuscitation protocol and monitoring

At the beginning of our clinical research we 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.

Patients were treated in two different fluid resuscitation regime. 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 $ScvO_2$ (CeVOX, Pulsion Medical Systems, Munich, Germany) was inserted via the central venous catheter (CVC). The correct position of the CVC was controlled by chest X-ray. 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[143]. 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.

In the HUO group, fluid resuscitation was guided by the following[52,144]: 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[145]. 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 using 20 ml cold (4°C) isotonic sodium chloride solution. Three readings were taken and the mean was recorded.

The fluid resuscitation in the ITBVI group was guided by 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}. The normal range for ITBVI is 850-1000 ml m^{-2}. By targeting the lower limit, we tried to avoid intravenous fluid overload. 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[52] 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

Inotrop and vasopressor administration were assessed. 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[146] and SOFA[147] scores were allocated. These scores were calculated in each patient daily after admission during the entire 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[148].

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 collected 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.

Blood 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 in a cooler on 4°C and processed in 6 hours after takeoff.

4.2.5.2.1. *Measurement of pro-, and antioxidant parameters*

Measurement of MDA with Ohakawa method[149]

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 attained plasma from ethylene diamine tetraacetic acid (EDTA) anticoagulated blood - centrifuged at 4,000 rpm for 10 min - and mixed with sodium-dodecyl sulphate, acid buffer and EDTA. Thiobarbiturate solution was added to the mixture and incubated for an hour at 90°C. After cooling, adding butanol and repeating centrifugation, the supernatant was measured with spectophotometry at 532 nm. We used tetrametoxipropane as a standard and MDA was expressed in μM/l.

Determination of ROS production in whole blood

Activated leukocytes, mainly neutrophils, are potential sources of ROS during inflammation. Free radical generating capacity of circulating leukocytes was assessed by measuring the amount of ROS in whole blood, with chemiluminescense (CL) method based upon the reaction of luminol with free radicals. To sum up, 20 μl EDTA anticoagulated blood was diluted in 1400 μl Dulbecco's modified Eagle's medium (DMEM) nutrient mixture of 37°C. 30 μl of 3-aminophtalhidrazide was added and the cuvette was immediately placed to Chrono-Log Whole Blood Lumi-aggregometer (Chrono-Log, Model 560, USA). The mixture was stirred and incubated at 37°C during measurement. After determining the spontaneous radical production, 50 μl phorbol-12 myristate-13 acetate (PMA) was injected into the cuvette and the resulting light output was recorded on a chart recorder. The peak value of free radical

production, the maximal rate of radical production were calculated from the recorded curve, and the results were related to the white blood cell (WBC) counts. The lag phase between PMA stimulation and the start of steep elevation in radical production was also determined.

Measurement of plasma MPO activity

MPO is a lysosomal enzyme that is found in neutrophil granulocytes and its plasma level elevates during inflammation. Plasma MPO level was obtained by adding 200 µl of plasma to 1 ml mixed solution (10.9 ml Na citrate, 100 µl o-Dianisidin, 1 ml H_2O_2 and 5 µl of 0.05% Triton-X-100). Incubation followed at 37°C for 5 min. After adding 1 ml of 35% perchloric acid to the solution, it was centrifuged for 10 min at 2,500 rpm and was measured at 560 nm.

Measurement of GSH in whole blood

GSH is a basic endogenous antioxidant, the level of which is reduced due to oxidative stress of various origins. A sample of 0.2 ml EDTA anticoagulated blood, haemolysed with 0.8 ml of distilled water, was mixed with 4 ml trichlore acetic acid (TCA) of 10% concentration. After centrifugation the supernatant was mixed with 4 ml TRIS buffer of pH 8.7. A colour reaction was induced with 100 µl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) followed by photometry at 412 nm. Using a standard GSH series for calibration, values were expressed in µM/l[150].

Measurement of plasma PSH level with Ellman's reagent

Plasma SH originates predominantly from plasma proteins and participate in the defence against oxidative stress. To determine sulfhydryl (SH) groups, 100 µl plasma, 100 µl Ellman's reagent (1 mM DTNB in methanol) and 800 µl EDTA containing TRIS buffer were mixed and photometry was performed at 412 nm. GSH standard series were used for calibration. The PSH amount was expressed in µM/l.

Determination of SOD enzyme activity in whole blood

SOD is an enzymatic endogenous antioxidant which catalyses the dismutation of the superoxide free radical. To determine SOD (mainly Cu/Zn-SOD) activity, 100 µl of EDTA anticoagulated blood was haemolysed with 900 µl distilled water and a mixture of ethanol and chloroform (2:1) was used to remove haemoglobin. Determination of the enzyme activity was based on the inhibition of the spontaneous oxidation of adrenaline to adrenochrome.

Spectrophotometric measurements were performed at 480 nm against sodium carbonate buffer (pH 10.2) blind. The values of SOD enzyme activity were given in IU/ml[151].

Determination of CAT enzyme activity in whole blood

CAT enzyme activity hemolysate was determined by the Aebi method[152]. CAT metabolizes H_2O_2 by reducing it to water and oxygen. This prevents the second generation of toxic intermediates. To determine CAT activity, 100 µl of EDTA anticoagulated blood was washed out with 900 µl distilled water. After centrifugation another washing process was performed. Absorbance of sample was measured at 240 nm on 37°C following administration of phosphate buffer, 10 µl red blood cell and 30 mmol H_2O_2. The values of CAT enzyme activity were given in BU/ml.

4.2.5.2.2. *Cytokine measurements*

Plasma was isolated from EDTA - anticoagulated blood samples by low-speed centrifugation at 4°C, and stored at -80°C until analyzed in a single batch at the end of the study. Human inflammation standards were diluted according to the manufacturer's instructions. 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. Principle of the test: six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-8, IL-1β, IL-6, IL- 10, TNFα and IL-12p70 proteins. The capture beads, R-Phycoerythrin-conjugated detection antibodies, and recombinant standards or test samples were incubated together to form sandwich complexes. FACS Calibur (BD Biosciences, USA) flow cytometer was used for acquisition of data. To determine the whole blood cytokine levels 4 ml heparin anticoagulated blood sample was incubated for 4 h on 37°C with 100 ml PMA solution containing 1 mg PMA. Following incubation and centrifugation the supernatant was stored on -80°C until cytokine assay. PMA is a receptor independent activator of circulating leukocytes. The rationale of its use was to measure the whole amount of pre-synthesized cytokines independently from any receptor up or down regulation.

4.2.5.2.3. *Measurement of 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. 200 µl of EDTA anticoagulated whole blood was mixed with 10 µl of mouse anti-human monoclonal antibodies CD11a, CD11b, CD18, CD49d, CD14, and CD97 (BD Pharmingen, San Diego, CA) conjugated with fluorescein isothiocyanate or phycoerythrin were used after 15 min incubation in dark at room temperature for immunofluorescence staining of leukocytes. Erythrocytes were haemolysed with diluted Becton Dickinson fluorescence activated cell sorter (BD FACS) Lysing Solution for 12 min. The leukocytes were washed twice in phosphate buffer solution (PBS), and finally resuspended in CellFIX solution. Cell immunofluorescence and light scatter data were acquired on a FACS-Calibur (BD Biosciences, San Jose, CA) flow cytometer and analyzed by Cellquest software. Mouse isotype controls (BD Biosciences, San Jose, CA) were used to determine the nonspecific background fluorescence. Binding of antibodies to leukocytes was quantified as the mean channel fluorescence in arbitrary units that exceeded nonspecific background fluorescence.

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 and occurrence of sepsis was significantly higher and ICU length of stay was significantly shorter. Demographic and clinical properties in the survivor- and non-survivor-groups are summarised in Table 2.

Table 2.: Demographic and clinical properties of patients. Data are presented as median and interquartile range

	Survivors (n=21)	Non-survivors (n=18)	p value
Age (years)	38 (32-45)	54 (43-70)	p<0.01
Male/female (n)	17/4	15/3	NS
Body mass index (kg m^{-2})	27 (22-32)	25 (21-30)	NS
Burned surface area (%)	42 (31-64)	49 (33-72)	NS
Deep burn injury (%)	34 (28-47)	38 (29-59)	NS
Inhalation injury (n)	15	16	NS
Flame burn injury (n)	12	9	NS
Blast burn injury (n)	3	4	NS
Scald burn injury (n)	6	5	NS
Occurrence of sepsis (n)	9	16	p<0.05
ICU length of stay (days)	39 (20-54)	23 (9-35)	p<0.01

ICU: intensive care unit; NS: non significant

The onset of sepsis was on the ninth (IQR, 7-12) day. The sources of infections and severity of sepsis are summarized in Table 3.

Table 3.: Sources of infections and severity of sepsis

	Survivors (n=21)	Non-survivors (n=18)
Wound infection	2	1
Pneumonia	1	3
Blood stream infection	2	7
Sepsis	1	0
Severe sepsis	2	4
Septick shock	2	7
Multiorgan failure	2	9

Hospital mortality rate was 46%. 11 of 18 patients died of septic complication in the ICU. Seven patients were discharged to the high-dependency unit of the surgical department. Three among them suddenly died because of heart insufficiency, 3 of them had to be readmitted to the ICU, 2 because of adult respiratory distress syndrome and 1 because of repeated septic shock.

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. Detection limits of the assay were taken from the Instruction Manual of CBA kit. IL-6 (Figure 5a.), IL-8 (Figure 5b.), IL-10 (Figure 5c.) concentrations were higher than the normal values in human plasma throughout observation. Pro-inflammatory cytokines IL-6 (Figure 5a.) and IL-8 (Figure 5b.) 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 (Figure 5c.).

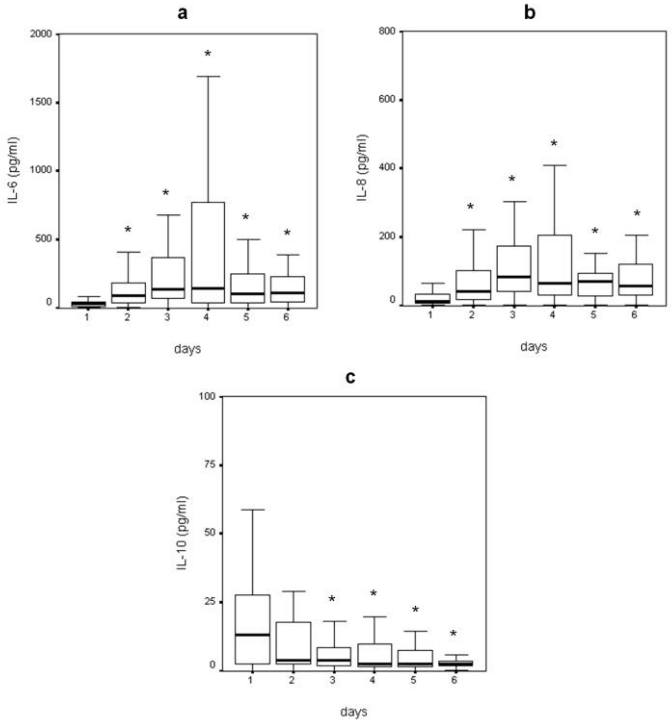


Figure 5.: Plasma levels of IL-6 (a), IL-8 (b) and IL-10 (c) in non-stimulated plasma samples on admission (day 1) and on the 5 consecutive days thereafter (days 2-6). Data are expressed as minimum, maximum, median and interquartile range. * Symbols indicate statistical differences comparing to day 1, *p<0.05

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 whole study period compared to day 1 (Figure 6a.). 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 (Figure 6b.).

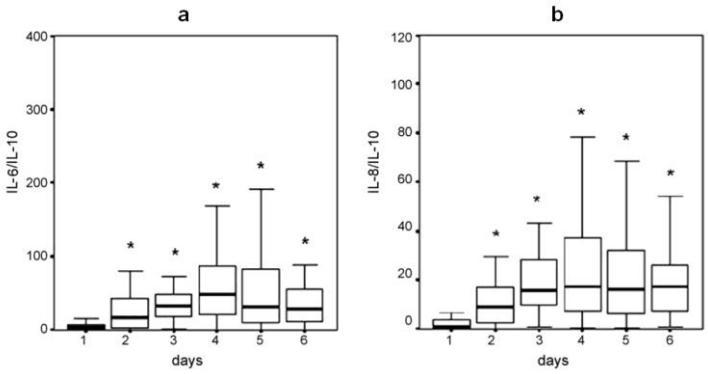


Figure 6.: IL-6/IL-10 (a), IL-8/IL-10 (b) ratios in non-stimulated plasma samples on admission (day 1) and on the 5 consecutive days thereafter (days 2-6). Data are expressed as minimum, maximum, median and interquartile range. * Symbols indicate statistical differences comparing to day 1, *p<0.05

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 the levels measured on admission (Figure 7a.). IL-8 showed a marked elevation after PMA stimulation. It reached the peak value on day 5 (Figure 7b.). 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 (Figure 7c.). 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 (Figure 7d.). 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 (Figure 7e.).

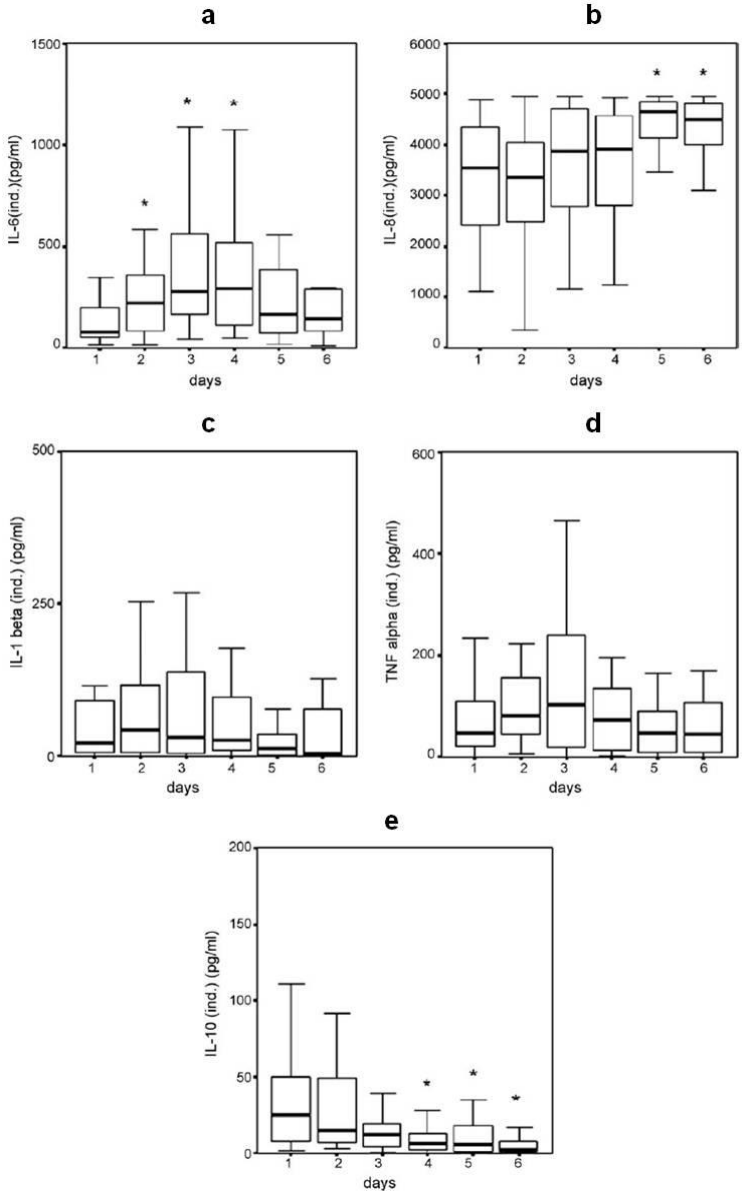


Figure 7.: Plasma levels of IL-6 (a), IL-8 (b), IL-1β (c), TNFα (d) and IL-10 (e) in stimulated plasma samples on admission (day 1) and on the 5 consecutive days thereafter (days 2-6). Data are expressed as minimum, maximum, median and interquartile range. * Symbols indicate statistical differences comparing to day 1, *$p<0.05$

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 (Figure 8a.). IL-8/IL-10 ratios increased during the whole study period. The highest value was detected on day 6. Significant ($p<0.05$) difference could be observed between day 1 and days 3-6 (Figure 8b.). 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 (Figure 8c.). TNFα/IL-10 ratios remained nearly on the same level during the

study period without any significant difference (Figure 8d.). 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.

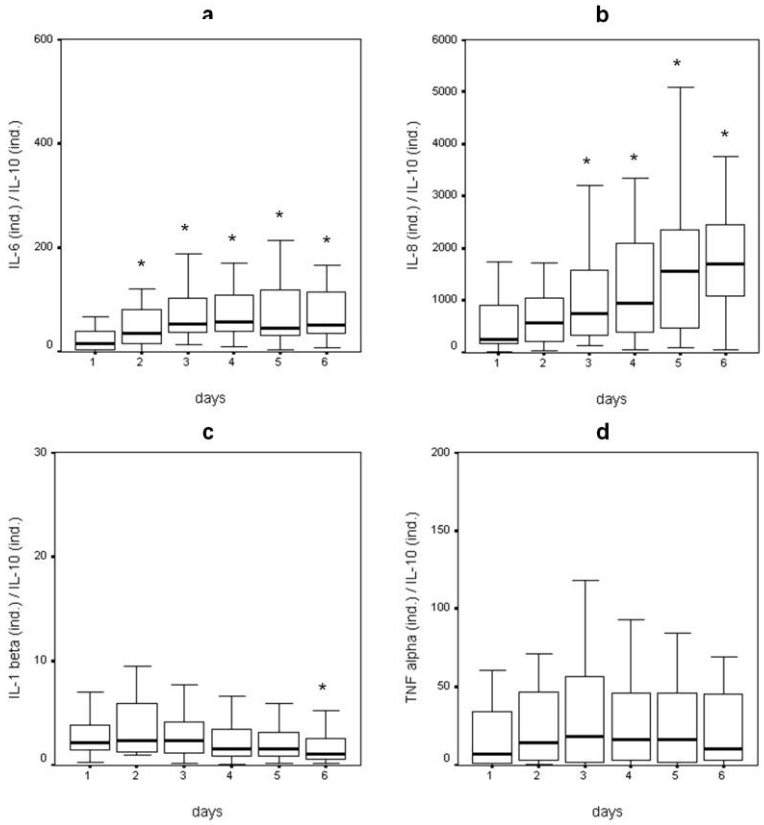


Figure 8.: Plasma levels of IL-6/IL-10 (a), IL-8/IL-10 (b), IL-1β/IL-10 (c) and TNFα/IL-10 (d) ratios in stimulated plasma samples on admission (day 1) and on the 5 consecutive days thereafter (days 2-6). Data are expressed as minimum, maximum,median and interquartile range. * Symbols indicate statistical differences comparing to day 1, *p<0.05

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

IL-6 (Figure 9a.), IL-8 (Figure 9b.), IL-10 (Figure 9c.) 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 ($p<0.001$) (Figure 9a.). 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 ($p<0.05$) (Figure 9b.). 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 ($p<0.05$)) (Figure 9c.).

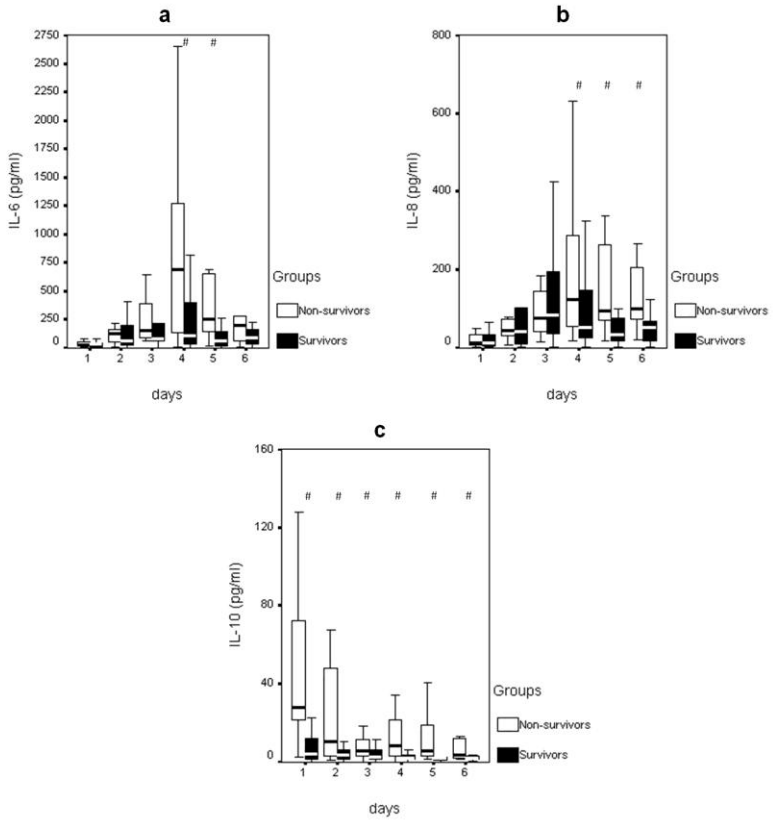


Figure 9.: Plasma levels of IL-6 (a), IL-8 (b) and IL-10 (c) in non-stimulated plasma samples on admission (day 1) and on the 5 consecutive days thereafter (days 2-6) in survivor and non-survivor patients. Data are expressed as minimum, maximum, median and interquartile range. # Symbols indicate intergroup statistical differences, #$p<0.05$

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 ($p<0.01$) (Figure 10a.). 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 (Figure 10b.).

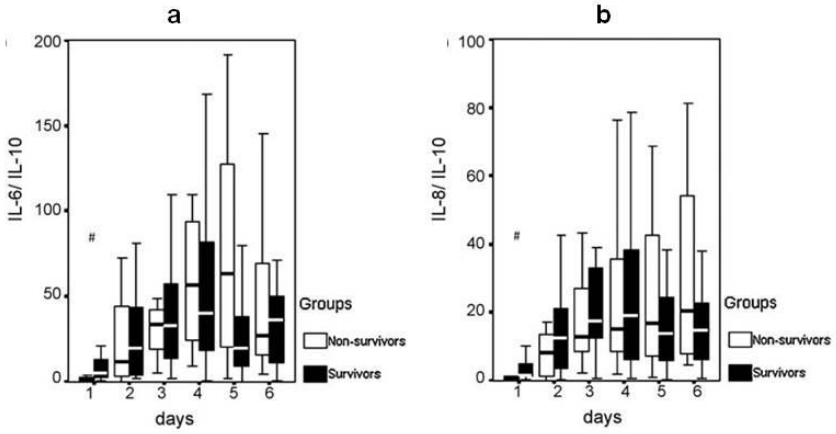


Figure 10.: IL-6/IL-10 (a), IL-8/IL-10 (b) ratios in non-stimulated plasma samples on admission (day 1) and on the 5 consecutive days thereafter (days 2-6) in survivor and non-survivor patients. Data are expressed as minimum, maximum, median and interquartile range. # Symbols indicate intergroup statistical differences, #$p<0.05$

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 (Figure 11a.) similarly to IL-8, which reached the peak value in survivors on day 5 in non-survivors on day 6 (Figure 11b.). 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 (Figure 11c.). 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 ($p<0.05$) (Figure 11d.). 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 ($p<0.05$)) (Figure 11e.).

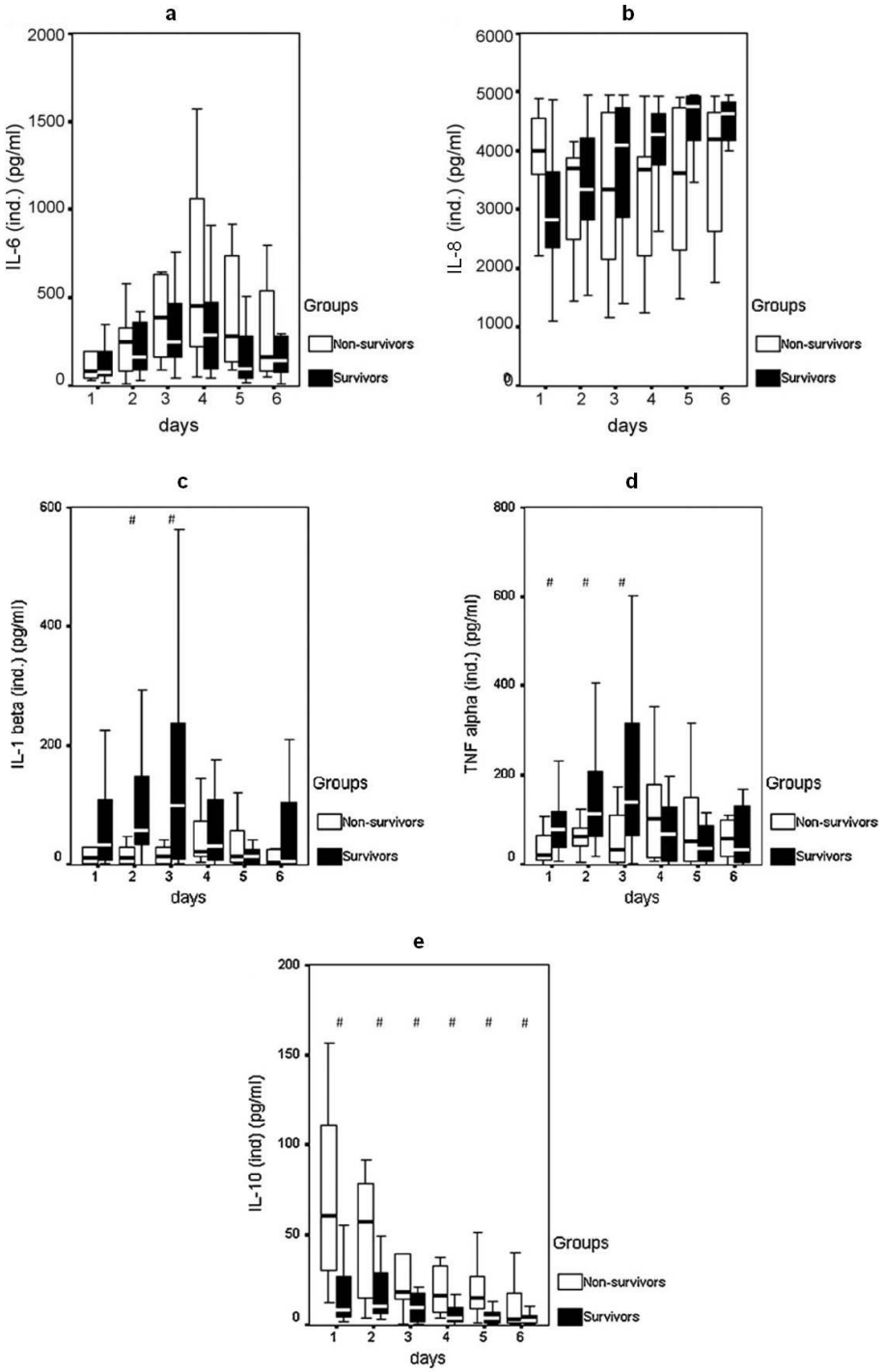


Figure 11.: Plasma levels of IL-6 (a) and IL-8 (b), IL-1β (c), TNFα (d) and IL-10 (e) in stimulated plasma samples on admission (day 1) and on the 5 consecutive days thereafter (days 2-6) in survivor and non-survivor patients. Data are expressed as minimum, maximum, median and interquartile range. # Symbols indicate intergroup statistical differences, #$p<0.05$

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 ($p<0.05$) (Figure 12a.). 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 (Figure 12b.). 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 (Figure 12c.). 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 (Figure 12d.).

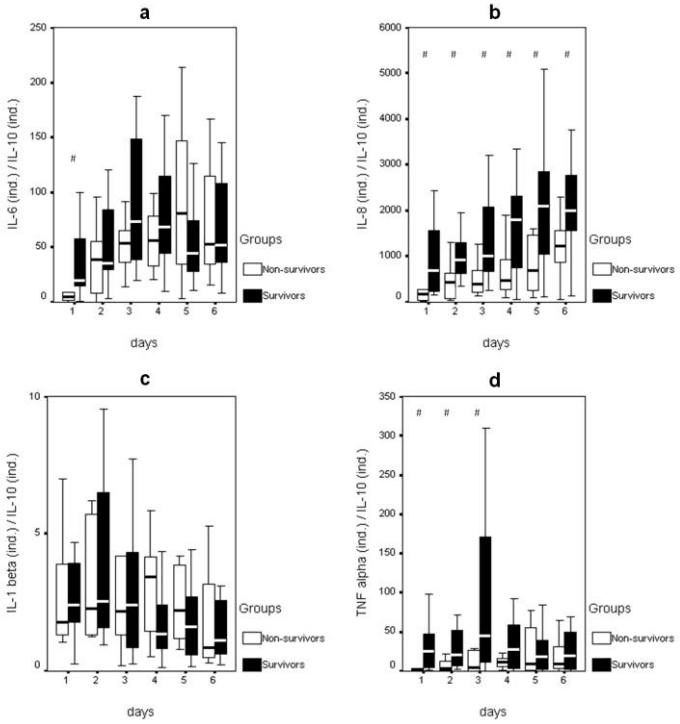


Figure 12.: Plasma levels of IL-6/IL-10 (a), IL-8/IL-10 (b) and IL-1β/IL-10 (c), TNFα/IL-10 (d) ratios in stimulated plasma samples on admission (day 1) and on the 5 consecutive days thereafter (days 2-6) in survivor and non-survivor patients. Data are expressed as minimum, maximum,median and interquartile range. # Symbols indicate intergroup statistical differences, #$p<0.05$

Changes in plasma HMGB1 concentration

Plasma HMGB1 concentration was markedly elevated on hospital admission in both survivors and non-survivors (Figure 13.). 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.

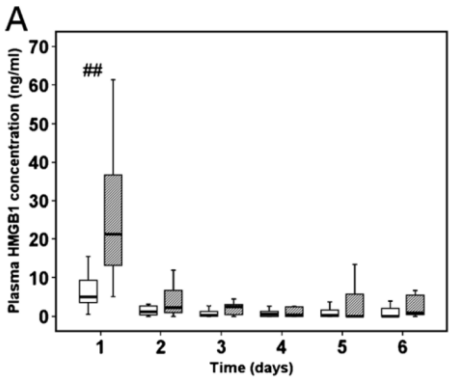


Figure 13.: Changes in plasma HMGB1 concentration in survivor (open box) and nonsurvivor (patterned box) patients. Data are expressed as median and interquartile range (standard 25th-75th percentile and 5th-95th percentile). $^{##}p<0.01$ survivors versus nonsurvivors

Positive correlations ($r=0.669$, $p<0.01$) were found between burned body surface and HMGB1 concentrations on admission (Figure 14.).

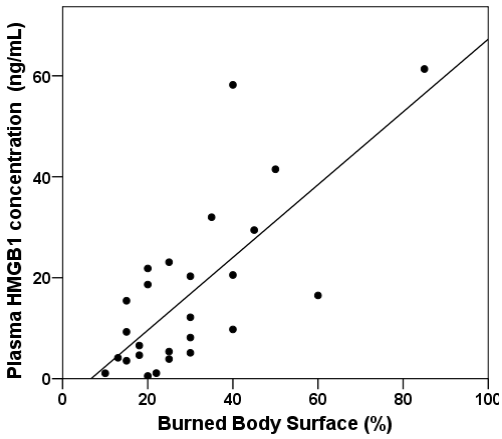


Figure 14.: Correlation between burned body surface and plasma HMGB1 concentration in patients on admission ($r=0.669$, $p<0.01$)

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) (Figure 15.).

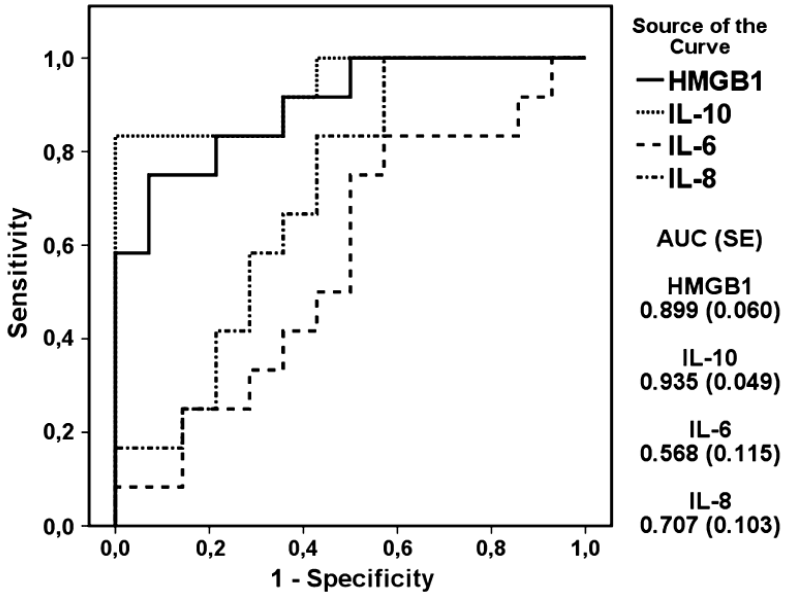


Figure 15.: Receiver Operating Characteristic (ROC) curve for IL-6, IL-8, IL-10 in non-stimulated plasma and HMGB1 measured on day 1 (on admission) in respect of ICU outcome

Areas under curves regarding the studied cytokines and cytokine ratios are summarised in Table 4.

Table 4.: Areas under curve of the studied cytokines and ratios

Cytokine	IL-6	IL-8	IL-10	$IL\text{-}6_{ind}$	$IL\text{-}8_{ind}$	$IL\text{-}10_{ind}$	$IL\text{-}1\beta_{ind}$	$TNF\alpha_{ind}$
AUC	0.556	0.581	0.869	0.513	0.731	0.888	0.331	0.281

Ratio	IL-6/IL-10	IL-8/IL-10	$IL\text{-}6/IL\text{-}10_{ind}$	$IL\text{-}8/IL\text{-}10_{ind}$	$IL\text{-}1\beta/IL\text{-}10_{ind}$	$TNF\alpha/IL\text{-}10_{ind}$
AUC	0.225	0.244	0.218	0.181	0.400	0.191

AUC: area under curve

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. (Fig. 15.).

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.

Comparison of cytokine levels in septic and nonseptic patients in non-stimulated plasma samples

Comparing septic and nonseptic patients, HMGB1 levels on admission were significantly higher in septic ones only on admission (Figure 16a.). IL-10 levels in septic patients were significantly higher compared with nonseptic ones during the whole study period (Figure 16b.). 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 (Figure 16c.) during our observation. There was a significant difference between septic and nonseptic patients in IL-8 on days 1, 4-6 (Figure 16d.), respectively.

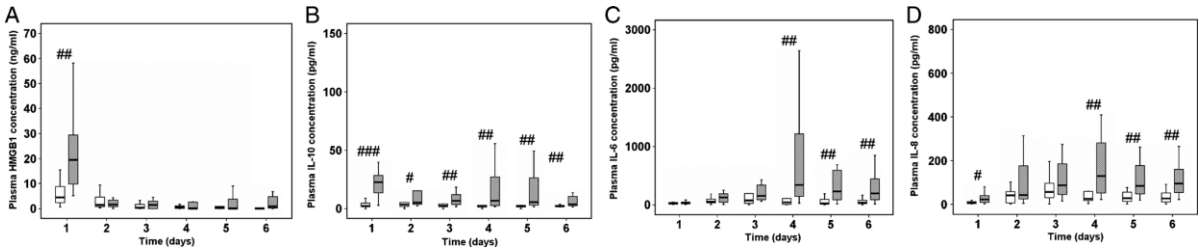


Figure 16.: Changes in plasma HMGB1 (A), IL-10 (B), IL-6 (C), and IL-8 (D) concentration in nonseptic (open box) and septic (patterned box) patients. Data are expressed as median and IQR (standard 25th-75th percentile and 5th-95th percentile). $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ nonseptic versus septic patients

Limitations of the study: our study holds some limitations. First of all it was powered to detecting prognostic values of IL-8 and IL-10 only. Regarding IL-1β, IL-12p70 and TNFα more sensitive tests would have been needed to measure their discriminating power. The use of PMA stimulation of leukocytes does not allow drawing conclusion regarding receptor specific changes. Our study was performed on circulating leukocytes but there is growing evidence that tissue cells are the primary sources of circulating cytokines. However, it is difficult to investigate their role in patients and the impact of activation of circulating immune cells has not been fully verified yet. This study is limited by a small study population of burned trauma patients. Our results demonstrated that an inflammatory reaction might be necessary for healing and an early anti-inflammatory excess may have a bad prognosis for the patients suffering from burn trauma.

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) requiring immediate intensive treatment (mechanical ventilation). Each patient survived the study period and all data were used for statistical analyses. There were no significant differences between groups regarding age, burn size, presence of inhalation injury and measured parameters on admission (Table 5.).

Table 5.: Demographic data and initial parameters of the patients

	HUO group (n= 15)	ITBVI group (n=15)	p value
Gender (M/F)	13/2	11/4	NS
Age (year)	56 (23-65)	58 (22-75)	NS
BBS (%)	42 (31-64)	44 (33-62)	NS
Deep burn injury	34 (28-56)	35 (27-58)	NS
Presence of inhalation injury	13	12	NS
ITBVI on admission (ml m^{-2})	780 (690-820)	790 (710-830)	NS
EVLWI on admission (ml kg^{-1})	5 (4-6)	5 (4-6)	NS
$ScvO_2$ on admission (%)	70 (67-72)	71 (67-73)	NS
Serum lactate level on admission (mmol l^{-1})	1.7 (1.4-2.2)	1.8 (1.6-2.3)	NS

All data were expressed as median and IQR. HUO: hourly urine output; ITBVI: intrathoracic blood volume index; M/F: male/female; BBS: burnt body surface; NS: non significant; EVLWI: extravascular lung water index; $ScvO_2$: oxygen saturation of the central venous hemoglobin

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 (Table 6.) 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. Table 6. shows a summary of the measured and calculated parameters during the study period.

Table 6.: First 6 days follow-up

Parameter	Group	1st 24 hours	2nd 24 hours	3rd 24 hours	4th 24 hours	5th 24 hours	6th 24 hours
$ScvO_2$ (%)	HUO	67 (62-70)	72 (65-78)	70 (62-73)	71 (66-77)	70 (64-78)	72 (68-76)
	ITBVI	72 (71-77)	74 (72-78)	73 (71-79)	73 (72-76)	71 (69-77)	74 (72-78)
		p<0.05	NS	NS	NS	NS	NS
ITBVI (ml m^{-2})	HUO	724 (592-823)	823 (753-867)	832 (778-856)	856 (731-878)	861 (772-889)	845 (767-869)
	ITBVI	823 (752-872)	844 (816-897)	848 (830-893)	859 (826-887)	856 (812-886)	849 (821-878)
		p<0.05	NS	NS	NS	NS	NS
Serum lactate (mmol l^{-1})	HUO	2.6 (1.5-3.7)	2.0 (1.4-3.2)	2.0 (1.3-3.1)	1.9 (1.2-3.1)	1.7 (1.1-2.6)	1.6 (1.0-2.3)
	ITBVI	2.3 (1.6-3.6)	2.1 (1.6-3.1)	1.9 (1.5-2.9)	1.8 (1.3-2.8)	1.6 (1.1-2.5)	1.5 (1.0-2.2)
		NS	NS	NS	NS	NS	NS
Urine output (ml kg^{-1} h^{-1})	HUO	0.8 (0.6-1.1)	0.9 (0.7-1.2)	0.9 (0.7-1.4)	1.0 (0.6-1.5)	1.1 (0.8-1.3)	1.1 (0.9-1.4)
	ITBVI	1.1 (0.9-1.3)	1.0 (0.8-1.1)	1.1 (0.7-1.5)	1.1 (0.8-1.3)	1.2 (0.9-1.4)	1.2 (0.9-1.5)
		P<0.05	NS	NS	NS	NS	NS
EVLWI (ml kg^{-1})	HUO	6 (5-7)	7 (6 -9)	7 (5 -9)	8 (6-10)	7 (5-10)	8 (6-10)
	ITBVI	6 (5-7)	6 (5-7)	7 (5-8)	6 (4-7)	7 (5-8)	6 (4-7)
		NS	NS	NS	NS	NS	NS
CI (l m^{-2})	HUO	2.8 (2.4-3.2)	3.8 (3.1-4.8)	3.9 (3.0-4.6)	3.7 (3.2-4.6)	3.8 (3.3-4.9)	3.7 (3.1-4.8)
	ITBVI	3.6 (3.3-3.9)	4.1 (3.3-5.6)	4.0 (3.4-5.4)	4.1 (3.5-5.4)	4.2 (3.6-5.4)	4.0 (3.4-5.2)
		p<0.05	NS	NS	NS	NS	NS

Table 6. continues on the next page

MODS	HUO	4.0 (2.0-5.0)	5.0 (3.5-6.0)	5.0 (3.8-6.3)	4.5 (3.4-5.0)	4.0 (3.0-4.8)	4.0 (2.8-4.6)
	ITBVI	3.5 (3.0-5.0)	3.5 (3.0-4.6)	3.0 (2.5-3.6)	3.5 (2.6-4.0)	3.0 (2.5-4.0)	3.0 (2.3-3.8)
		NS	p<0.05	p<0.05	NS	NS	NS
Patients requiring vasopressor support	HUO	3	6	7	6	5	4
	ITBVI	2	4	5	4	3	3
		NS	NS	NS	NS	NS	NS
Daily fluid requirement (ml body weight kg^{-1} BBS^{-1})	HUO	4.5 (4.1-5.4)	2.5 (1.8-2.7)	1.4 (1.0-1.8)	1.3 (0.9-1.6)	1.4 (0.8-1.7)	1.3 (0.8-1.6)
	ITBVI	5.4 (4.2-6.6)	2.0 (1.6-2.3)	1.3 (0.9-1.6)	1.3 (0.8-1.6)	1.3 (0.7-1.6)	1.2 (0.7-1.4)
		p<0.05	p<0.05	NS	NS	NS	NS

Data are expressed as median and interquartile range. Median values were calculated taking all measurements of all patients of a group on the same day. $ScvO_2$: oxygen saturation of the central venous hemoglobin; ITBVI: intrathoracic blood volume index; HUO: hourly urine output; CI: cardiac index; EVLWI: extravascular lung water index; MODS: multiple organ dysfunction score; NS: non significant; BBS: burnt body surface

Clinical data are summarized in Table 7.

Table 7.: Summary of clinical data

	HUO group (n=15)	ITBVI group (n=15)	p value
Hospital mortality	5/15	3/15	NS
Mechanical ventilation (days)	28 (22-35)	26 (18-32)	NS
Septic episodes during hospital stay	7	6	NS
MOF during hospital stay	4	2	NS
Surgical procedures during hospital stay	43	44	NS
Intra-abdominal compartment syndrome	0/15	0/15	NS
ICU stay (days)	41 (37-58)	39 (35-59)	NS
Hospital stay	72 (61-79)	69 (60-75)	NS

HUO: hourly urine output; ITBVI: intrathoracic blood volume index; ICU: intensive care unit; MOF: multiple organ failure; NS: non significant

Significant differences were not found between groups regarding hospital mortality, hospital and ICU stay, days on ventilator, occurrence of sepsis and MOF. 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**.**

The effect of fluid resiscitation method on 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 whole study period. IL-6 (Figure 17a.), IL-8 (Figure 17b.), IL-10 (Figure 17c.) 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 ($p<0.05$, Figure 17a.) whereas elevated levels of IL-10 could be observed in the HUO group (Figure 17c.) on days 4-6 ($p<0.05$). The IL-6/IL-10 ratio on days 2-3 (Figure 18a.), and the IL-8/IL-10 ratio on days 3-5 (Figure 18b.) were significantly higher in the ITBVI group ($p<0.05$).

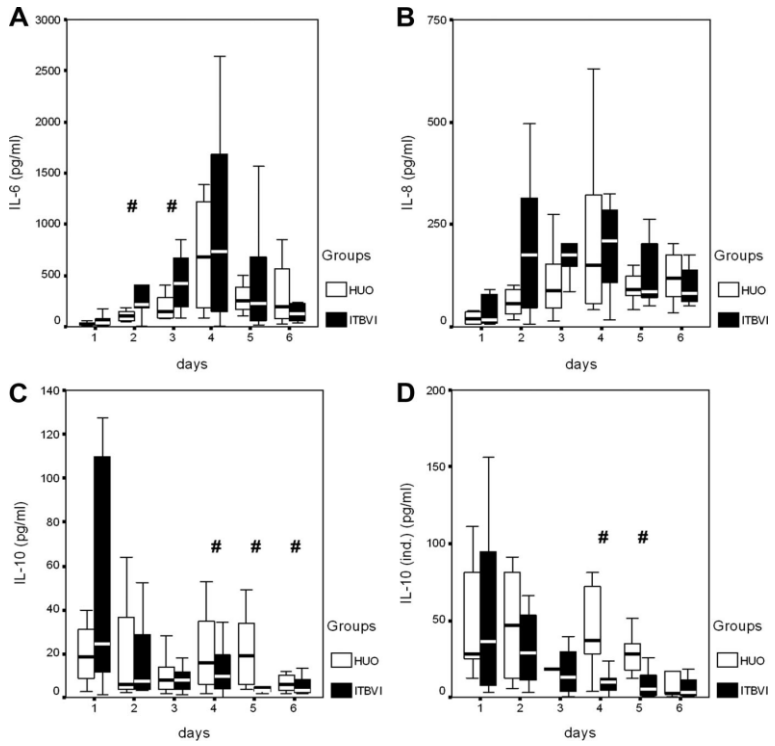


Figure 17.: Levels of IL-6 (a), IL-8 (b) and IL-10 (c) in HUO and ITBVI groups of patients in non-stimulated plasma samples and levels of IL-10 (d) in the stimulated plasma samples. Data are expressed as minimum, maximum, median and interquartile range. HUO = hourly urine output group, ITBVI = intrathoracic blood volume index group. # = $p<0.05$ HUO group vs. ITBVI group

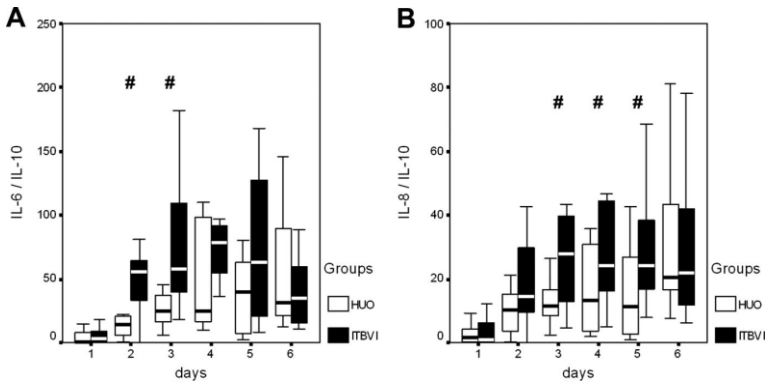


Figure 18.: IL-6/IL-10 (a) and IL-8/IL-10 (b) ratios in HUO and ITBVI groups of patients in non- stimulated plasma samples. Data are expressed as minimum, maximum, median and interquartile range. HUO = hourly urine output group, ITBVI = intrathoracic blood volume index group. # = $p<0.05$ HUO group vs. ITBVI group

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 (Figure 17d.) were observed on days 4-5 ($p<0.05$) with higher levels in the HUO group.

The effect of fluid resuscitation method on leukocyte adhesion molecule expression

The granulocyte CD11a (Figure 19a.) levels were significantly higher on the second day in the HUO group ($p<0.05$) compared to the ITBVI group. CD11b levels (Figure 19b.) 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 (Figure 19c.) and CD97 (Figure 19d.).

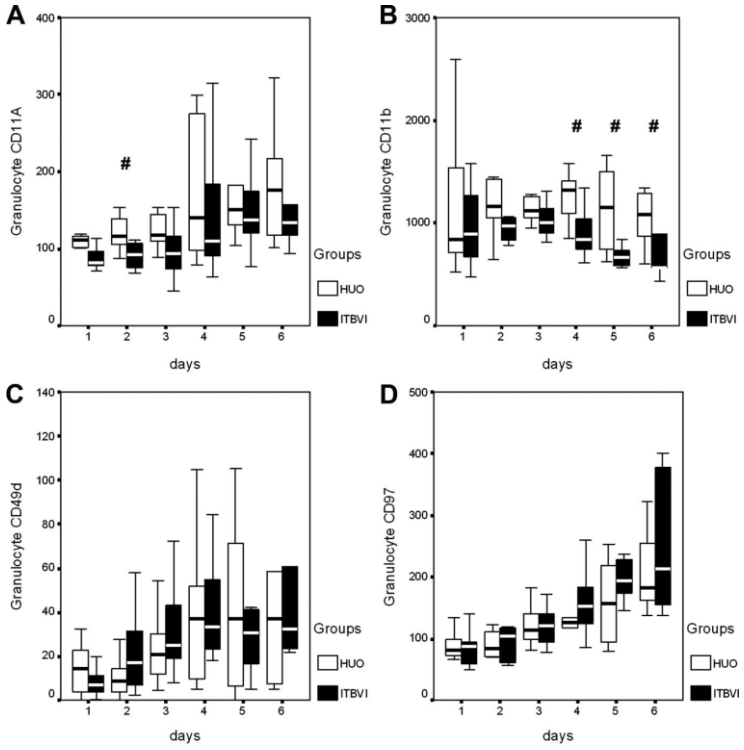


Figure 19.: Granulocyte CD11a (a), CD11b (b), CD49d (c) and CD97 (d) expressions in HUO and ITBVI groups of patients. Data are expressed as minimum, maximum, median and interquartile range. HUO = hourly urine output group, ITBVI = intrathoracic blood volume index group. # = $p<0.05$ HUO group vs. ITBVI group

Lymphocyte CD11a (Figure 20a.) on days 5-6, lymphocyte CD11b (Figure 20b.) on days 3-6, lymphocyte CD49d (Figure 20c.) on days 2-6 and lymphocyte CD97 (Figure 20d.) on day 6 were significantly lower ($p<0.05$) in the ITBVI group than in the HUO group whereas CD18 (data not shown) did not show significant difference between groups.

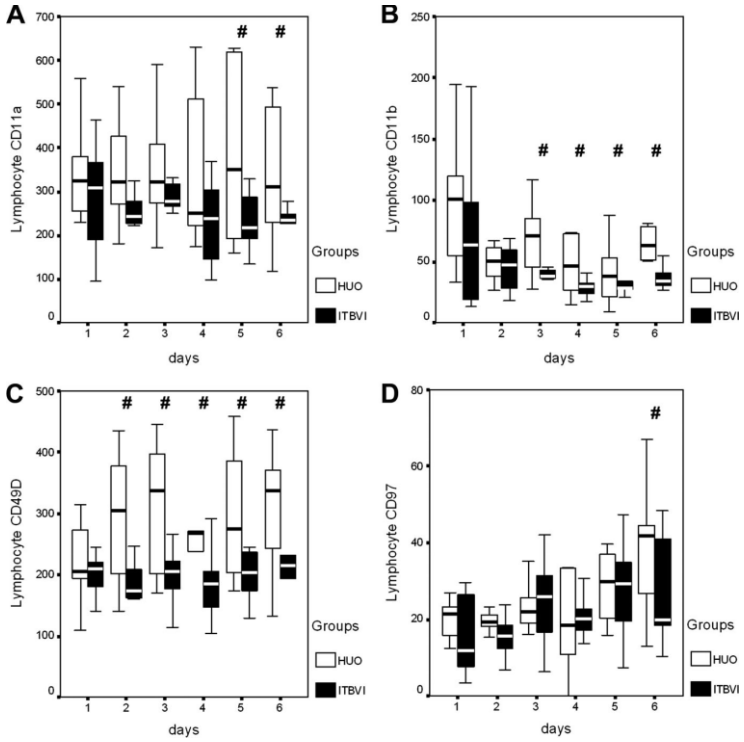


Figure 20.: Lymphocyte CD11a (a), CD11b (b), CD49d (c) and CD97 (d) expressions in HUO and ITBVI groups of patients. Data are expressed as minimum, maximum, median and interquartile range. HUO = hourly urine output group, ITBVI = intrathoracic blood volume index group. # = $p<0.05$ HUO group vs. ITBVI group

Comparing the HUO group to the ITBVI group, monocyte CD11a (Figure 21a.), CD11b (Figure 21b.), CD18 (Figure 21c.) levels showed a significant decrease ($p<0.05$) in ITBVI group on days 4-6. The CD14 (Figure 21d.) level was significantly lower in the ITBVI than in the HUO group on days 3-5 ($p<0.05$), whereas CD49d and CD97 (data not shown) did not differ significantly between groups.

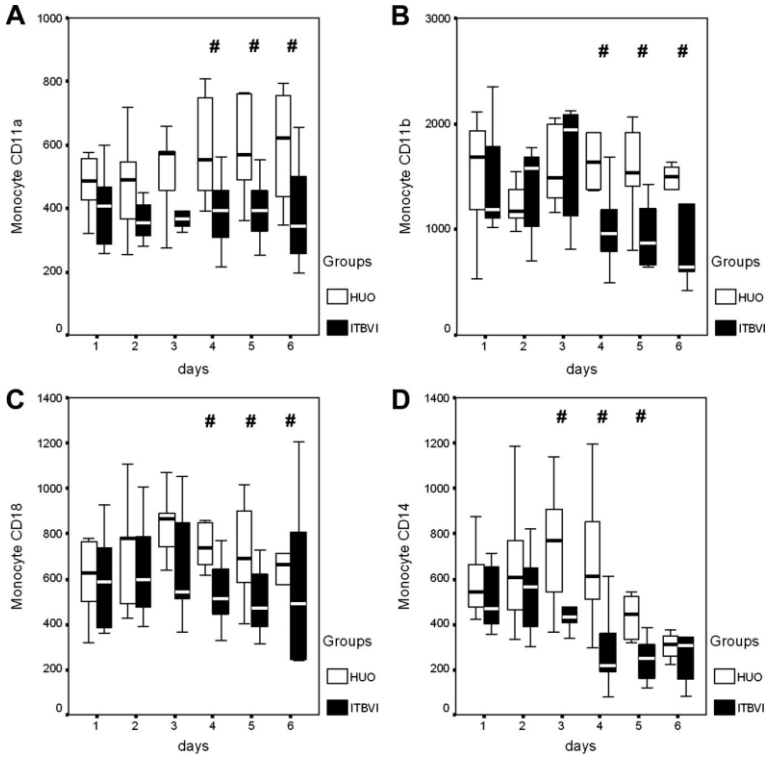


Figure 21.: Monocyte CD11a (a), CD11b (b), CD18 (c) and CD14 (d) expressions in HUO and ITBVI groups of patients. Data are expressed as minimum, maximum, median and interquartile range. HUO = hourly urine output group, ITBVI = intrathoracic blood volume index group. # = $p<0.05$ HUO group vs. ITBVI group

Limitations of the study: we were only able to measure TNFα and IL-1β levels after PMA stimulation. Unfortunately, the detection limits of our CBA kit were slightly higher than TNFα and IL-1β concentrations had been reported in postburn patients[153]. Therefore, using a more sensitive method would have been beneficial. CD markers of circulating leukocytes were well detected. Most of the circulating leukocytes were newly released from the bone marrow following trauma and it was not fully investigated how they might reflect the CD expression of adhered leukocytes. Moreover, our study is underpowered for drawing conclusion on mortality and clinical outcome parameters except MOD scores. 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 soluble cytokines towards anti-inflammatory imbalance and expression of leukocyte surface markers in burned patients.

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 of patients

30 patients were involved in this prospective randomised study. There were no significant differences between groups regarding age, burned surface area, extent of deep burn, and occurrence of inhalation injury, mechanism of burn and in calculated organ function scores. Demographic and initial clinical properties in the NAC and standard groups are summarised in Table 8.

Table 8.: Demographic and clinical properties of patients on admission. Data are presented as median and interquartile range

	NAC (n=15)	Standard (n=15)	p value
Age (years)	55 (45-74)	49 (33-60)	NS
Male/female	12/2	11/3	NS
Burned surface area (%)	50 (44-56)	44 (40-50)	NS
Deep burn (%)	35 (30-40)	33 (30-40)	NS
Inhalation (n)	11	9	NS
Flame burn (n)	5	7	NS
Blast burn (n)	9	6	NS
Scald burn (n)	1	2	NS
PCT	0.3 (0.1-1.8)	0.4 (0.1-3.5)	NS
SOFA	4 (1-9)	5 (4-7)	NS
MODS	4 (2-9)	5 (4-8)	NS
ITBVI (ml m^{-2})	780 (690-820)	790 (720-810)	NS

NAC: N-acetylcysteine; PCT: procalcitonine; SOFA: sequential organ failure assessment; MODS: multiple organ dysfunction score; ITBVI: intrathoracic blood volume index; NS: non-significant

Changes in leukocyte count

WBC 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 (Figure 22a.). 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 (Figure 22b.) and that of the lymphocyte ratio was highest (Figure 22c.) 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 (Figure 22d.) in both groups.

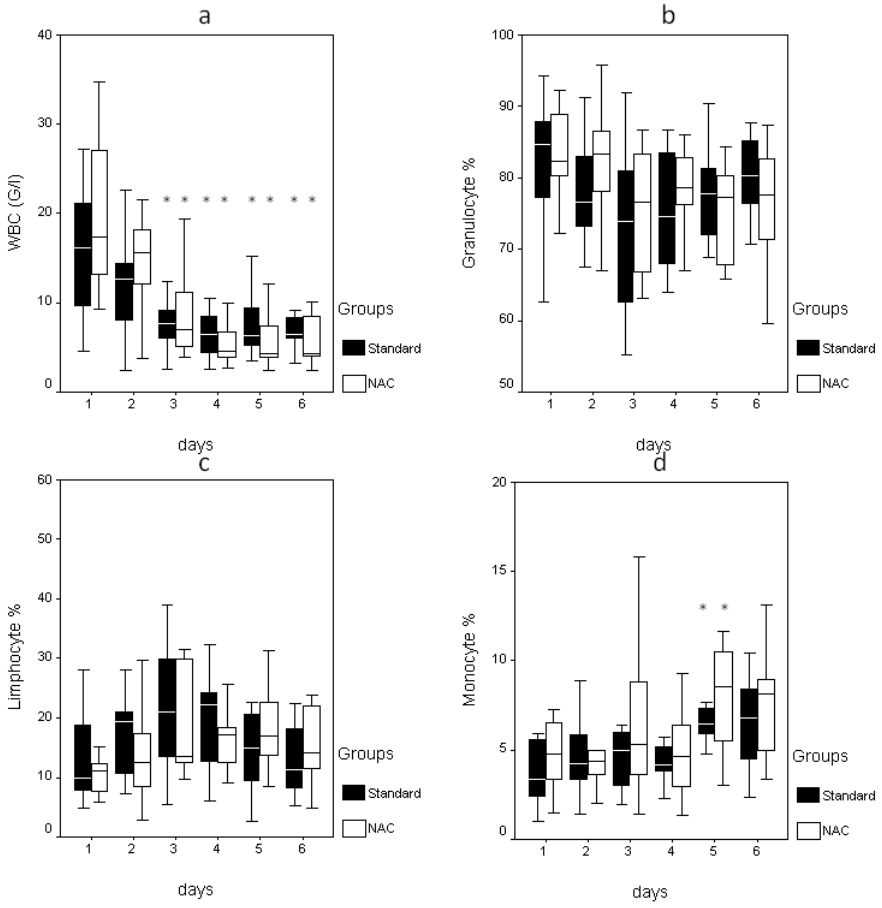


Figure 22.: Changes in white blood cell count (a), relative number of granulocytes (b), lymphocytes (c), and monocytes (d) in patients with standard therapy and NAC supplementation. Data are expressed as minimum, maximum, median and interquartile range. NAC = N-acetylcysteine, *Symbols indicate intra-group differences comparing to day 1, * = $p<0.05$

The effect of NAC treatment on the oxidative stress markers

Changes in the pro-oxidant markers

Plasma MDA level increased significantly on day 2 in both groups, but without any significant differences between groups (Figure 23a.). Plasma MPO activity increased on days 5-6 in both groups but the elevation was not significant. No significant differences were found between groups (Figure 23b.). Maximal value and rate (Figure 23c, 23d.) of ROS production showed significant elevation in both groups from day 4 compared to the day 1 values, without any significant differences between groups (Figure 23c, 23d).

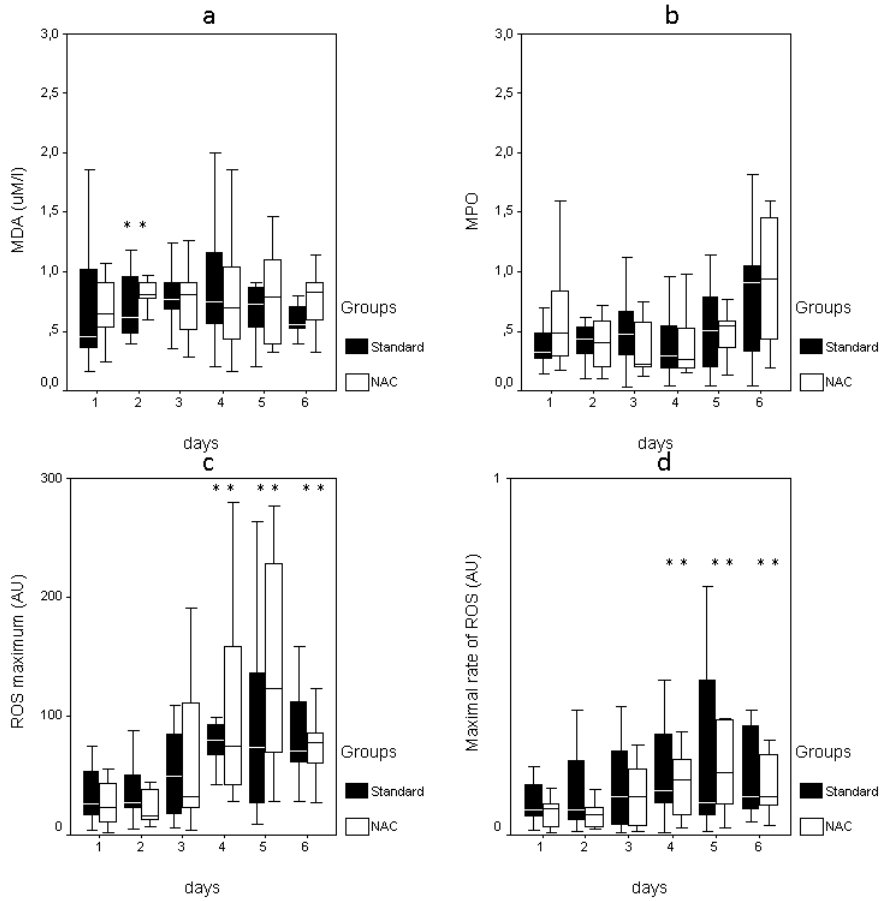


Figure 23.: Changes in plasma malondialdehyde concentration (a), plasma myeloperoxidase enzyme activity (b), maximal value (c) and maximal rate (d) of ROS production in whole blood of patients with standard therapy and NAC supplementation. Data are expressed as minimum, maximum, median and interquartile range. NAC = N-acetylcysteine, *Symbols indicate intra-group differences comparing to day 1, * = $p<0.05$

Changes in the levels of 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 (Figure 24a.). 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 (Figure 24b.). 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 (Figure 24c.). 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 (Figure 24d.).

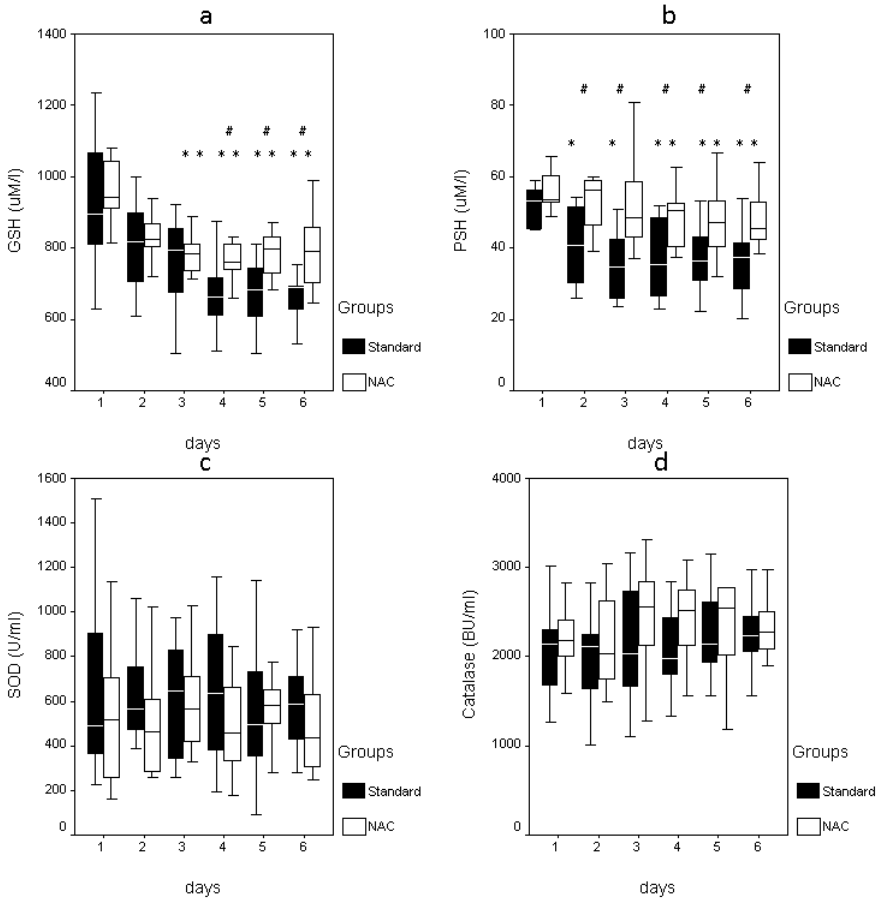


Figure 24.: Changes in GSH concentration in hemolysate (a), concentration of plasma SH groups (b), superoxide dismutase (c), and catalase (d) enzyme activity in hemolysate of patients with standard therapy and NAC supplementation. Data are expressed as minimum, maximum, median and interquartile range. NAC = N-acetylcysteine, *Symbols indicate intra-group differences comparing to day 1, * = $p<0.05$. #Symbols indicate differences between NAC and standard group, # = $p<0.05$

The effect of NAC treatment on non-stimulated (native) plasma cytokine levels

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 (Figure 25a.). 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 (Figure 25b.). 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 (Figure 25c.).

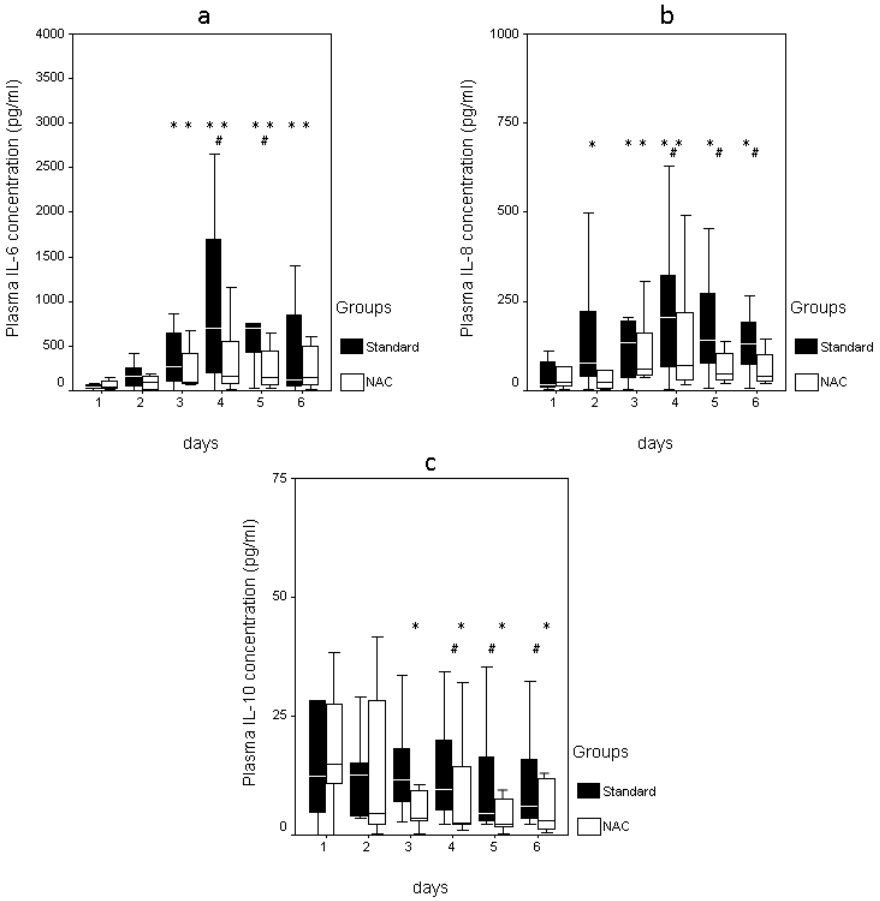


Figure 25.: Changes in IL-6 (a), IL-8 (b), and IL-10 (c) concentration (non-stimulated plasma samples) in patients with standard therapy and NAC supplementation, NAC = N-acetylcysteine. *Symbols indicate intra-group differences comparing to day 1, * = $p<0.05$. #Symbols indicate differences between NAC and standard group, # = $p<0.05$

The effect of NAC treatment on leukocyte cell surface marker expression

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 (Figure 26a.). CD11b showed a slight decreasing tendency from day 5, without any significant difference between groups or compared to day 1 (Figure 26b.). 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 (Figure 26c.). 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 (Figure 26d.). 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 (Figure 26e.).

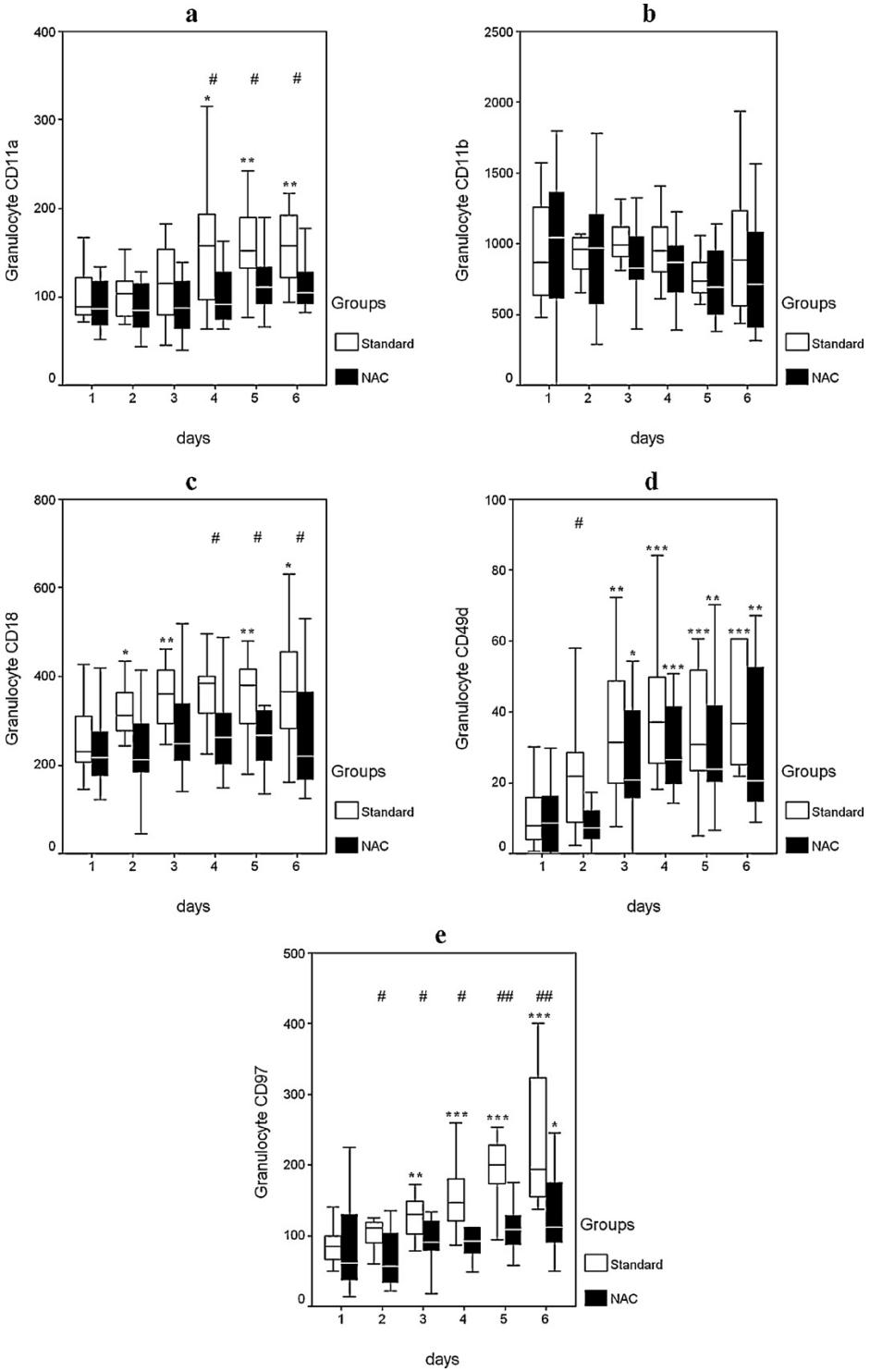


Figure 26.: Granulocyte CD11a (a), CD11b (b), CD18 (c), CD49d (d) and CD97 (e) expressions in patients with standard therapy and NAC supplementation. Data are expressed as minimum, maximum, median and interquartile range. NAC: N-acetylcysteine, *Symbols indicate intragroup differences comparing to day 1, * p<0.05, ** p<0.01, *** p<0.001. #Symbols indicate differences between NAC and standard group, # p<0.05, ## p<0.01

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 (Figure 27a.). CD11b did not show significant changes, either in standard or in NAC group, or significant differences between groups (Figure 27b.). CD18 levels decreased in both groups, although more markedly in NAC group, without significant differences compared to day 1 and between groups (Figure 27c.). 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 (Figure 27d.). 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 (Figure 27e.).

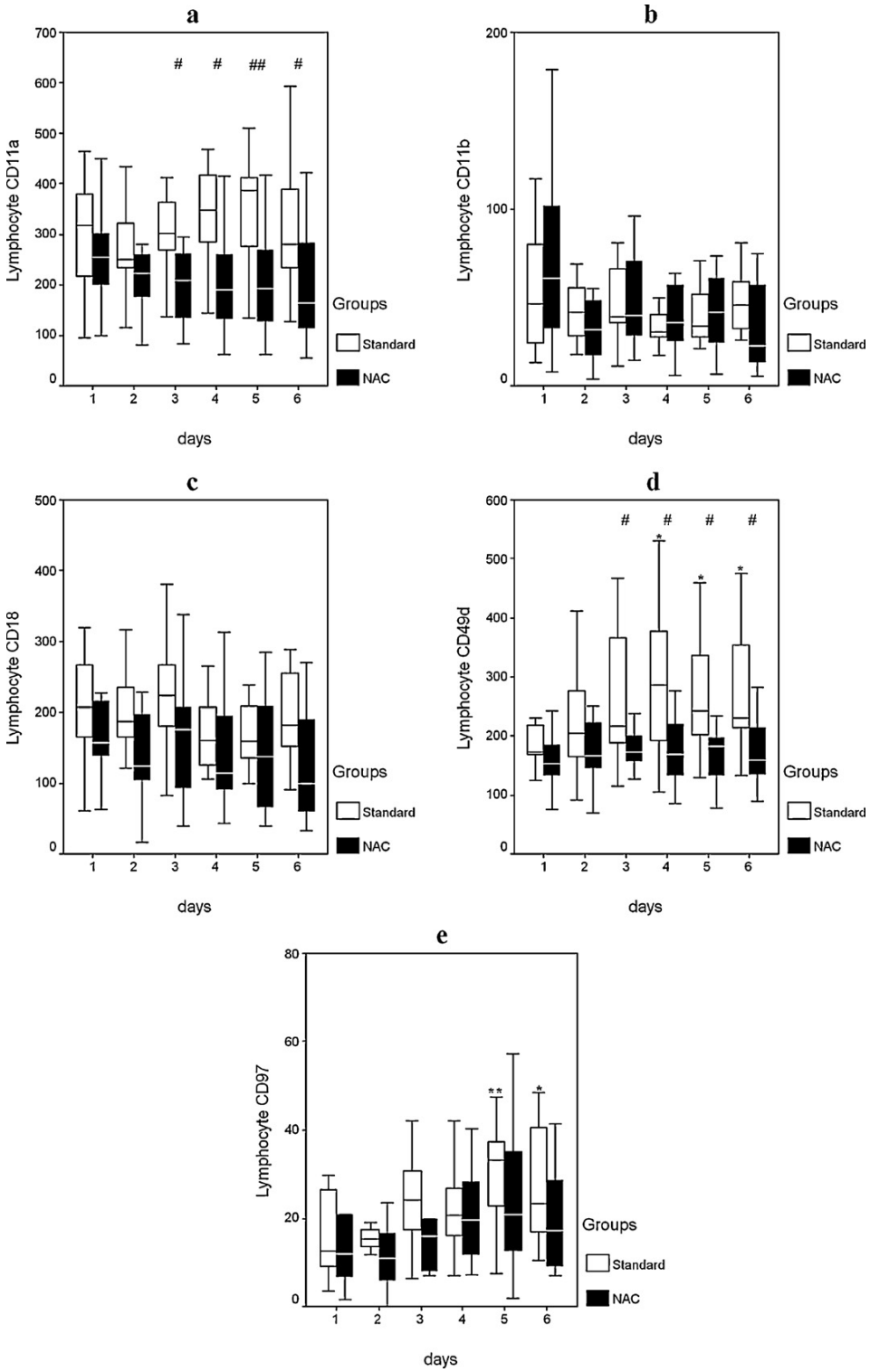


Figure 27.: Lymphocyte CD11a (a), CD11b (b), CD18 (c), CD49d (d) and CD97 (e) expressions in patients with standard therapy and NAC supplementation. Data are expressed as minimum, maximum, median and interquartile range. NAC: N-acetylcysteine, *Symbols indicate intragroup differences comparing to day 1, * $p<0.05$, ** $p<0.01$. #Symbols indicate differences between NAC and standard group, # $p<0.05$, ## $p<0.01$

Monocyte CD11a failed to show any significant differences compared to day 1 or between groups (Figure 28a.), similarly to CD11b (Figure 28b.) and CD18 levels (Figure 28c.). 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 (Figure 28d.). 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 (Figure 28e.). 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 (Figure 28f.).

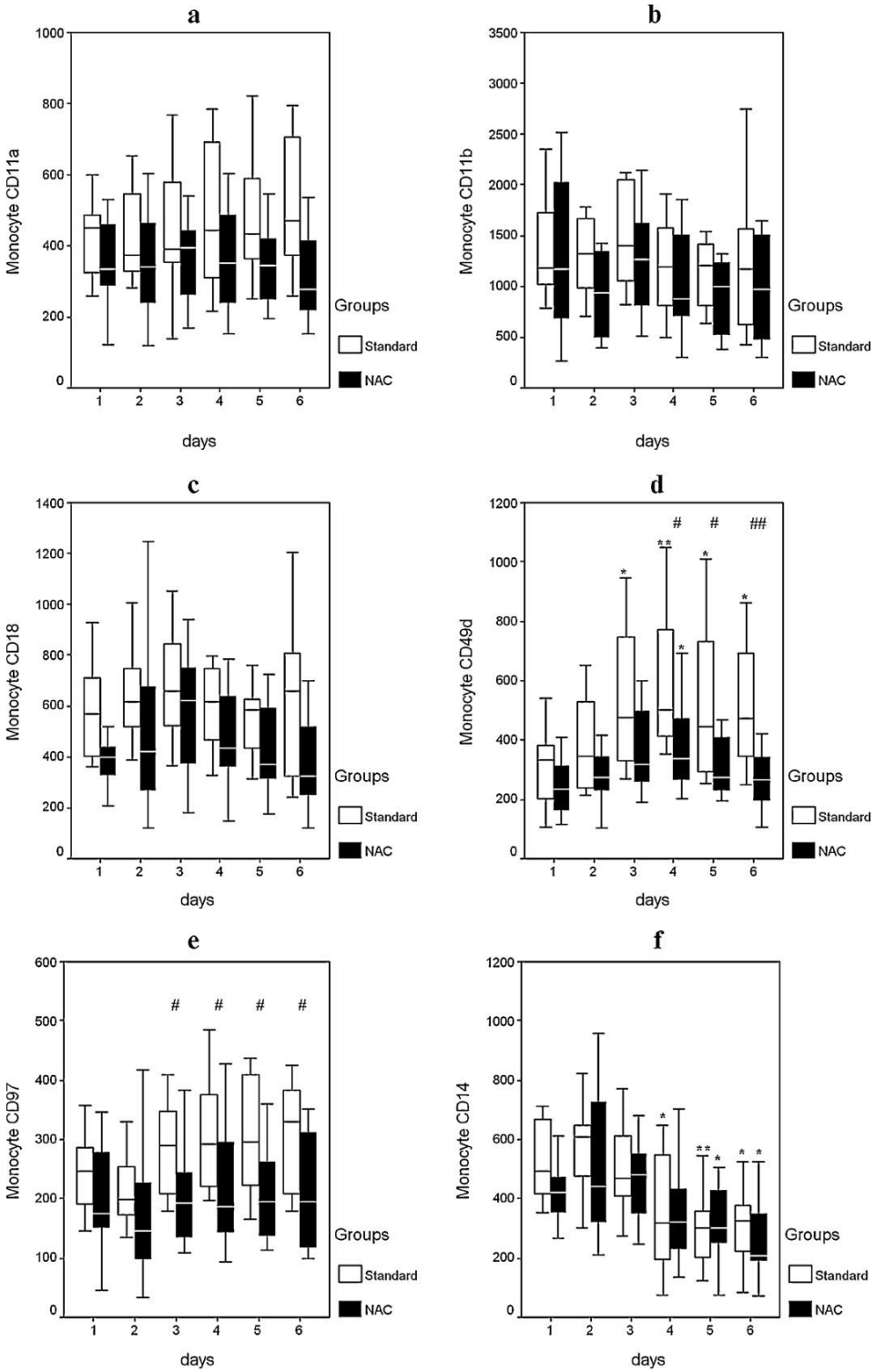


Figure 28.: Monocyte CD11a (a), CD11b (b), CD18 (c), CD49d (d), CD97 (e) and CD14 (f) expressions in patients with standard therapy and NAC supplementation. Data are expressed as minimum, maximum, median and interquartile range. NAC: N-acetylcysteine, *Symbols indicate intragroup differences comparing to day 1, * $p<0.05$, ** $p<0.01$. #Symbols indicate differences between NAC and standard group, # $p<0.05$, ## $p<0.01$

Clinical 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 (Figure 29.).

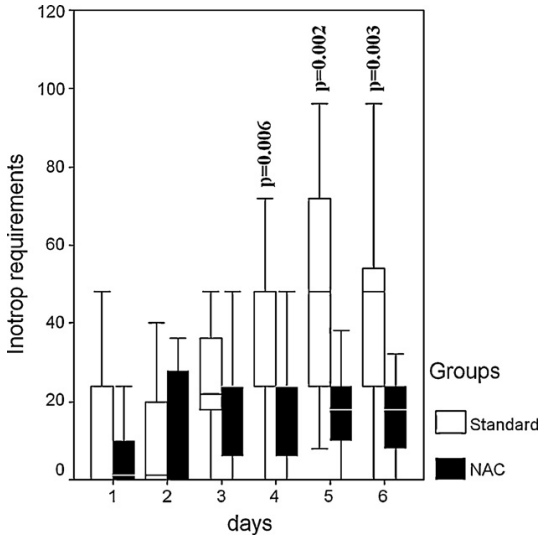


Figure 29.: Inotropic and vasopressor drug requirements in patients with standard therapy and NAC supplementation. NAC: N-acetylcysteine

There were no significant differences in preload of patients reflected in ITBVI during the whole study period (data not shown). Daily SOFA and MOD scores did not show any significant difference between NAC and standard group during the study period (data not shown). 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 (Table 9.).

Table 9.: Clinical outcome parameters

	NAC group (n=15)	Standard group (N=15)	p value
Days on respirator	29 (21-37)	26 (17-34)	NS
ICU length of stay (days)	41 (32-56)	39 (33-58)	NS
Mortality (n)	4	6	NS

NAC: N-acetylcysteine; ICU: intensive care unit; NS: non significant

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 (Table 9.).

Limitations of the study: 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.

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 is 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[154]. Although most of the studies report an elevation in pro- and anti-inflammatory cytokines following burn injury[155],[156] 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 although, De Bandt and associates found elevated levels of TNFα only on day 7 and elevated levels of IL-1ß were only rarely detected[101]. Fukushima and associates suggested that serum levels of IL-1 are not increased after burn injury[103]. Carsin and associates found elevated levels of TNFα in only one out of forty patients[157].

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. Examining the IL-10 levels in 8 survivors and 7 non-survivors the authors found that IL-10 levels showed a significantly higher peak value

only before the death of the patients compared to survivors[155]. Ozbalkan et al.[158] 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[82,110] , and confirm that an early shift can be observed towards anti-inflammatory cytokine production which makes the patient susceptible to infections. In the initial phase of trauma or burn injury macrophages are the main sources of interleukins[82,159,160] and the suppression of T helper type 1 (TH1) cells plays a role in this shift later on. 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. Previous observations proved that elevated IL-10 levels are associated with susceptibility to infection and in this way supports our findings. The importance of early elevation of IL-10 was underlined by the study of Lyons et al.[108] who have proven that immediate anti-IL-10 antibody treatment after injury could improve the survival of burned mice suffering from sepsis caused by cecum ligation and puncture. Administration of anti-IL-10 antibody on the third day after injury could decrease the level of circulating IL-10 but it could not increase survival. These data underline the prognostic value of IL-10 level measured on admission.

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[80]. 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. Although most publications reported an elevated level of TNFα after burn injury[94]. Finnerty et al. did not find an elevated level of TNFα similarly to our results[80]. Moreover, Agay et al. found that TNFα was undetectable in all biological samples taken from the 40% TBSA-burned rats (blood, liver, lung) and the serum level of IL-1β remained below the level of detection but an elevated level of IL-1β could be detected in the central nervous system and lung tissue[104]. According to the results of Drost et al.[161] TNFα was also undetectable in most plasma samples in burned patients, but they measured elevated IL-1β levels contrary to our result. 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[41], 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[155]. Elevated levels of TNFα, IL-1β, IL-6, IL-8 and IL-10 could be detected in the stimulated samples similarly to other studies. In the literature no study has been found examining parallel the cytokine levels in stimulated and non-stimulated blood samples in burned patients. Using of stimulated and non-stimulated samples may be the underlying cause of the conflicting results. On the other hand the detection limits of the kits are different. Finnerty et al. reported elevated TNFα, IL-12p70 and IL-6 levels in survivors and non-survivors on admission, but the measured values were around our detection limits[152]. In the cited study using multiple logistic regression elevation of IL-6 and IL-12p70 and decreased TNFα levels on admission were the best predictors of mortality. In contrast to this study in our work IL-10 on admission showed a good predictive value. The AUC-s in our study and in the study reported by Finnerty et al. were nearly similar. Significantly lower TNFα levels and TNFα/IL-10 ratios were found in the non-survivors in our study. These findings may support the results of Finnerty et al. regarding the predictive value of lower TNFα levels. The cytokine network orchestrates the inflammatory processes. In an extremely elevated concentration proinflammatory cytokines can modulate the function of organs, while dominant anti-inflammatory ones may counteract them. The balance between pro- and anti-inflammatory cytokines may be essential for appraising the genuine effect of different cytokines[162]. In our study the ratio of pro- and anti-inflammatory cytokines was higher in survivors till day 3 following trauma, and gradually decreased thereafter, while it showed a step-by-step elevation in non-survivors later on.

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[125,126,127,154,163,164]. Several experimental data confirm that burn injury induces an elevation in plasma HMGB1 concentration[128,129]. Using the same commercially available ELISA system, plasma median HMGB1 concentrations were less than 2 ng mL^{-1} in healthy humans[127,161]. Our results confirmed a marked elevation in HMGB1 after burn injury, as recently reported by Dong and associates[130]. In our study, plasma HMGB1 levels were markedly elevated in the very early hours after burn trauma. Using the same assay system, Peltz and associates[127] have recently published that plasma HMGB1 was elevated more than 30-fold above healthy controls within 1 h of trauma injury and peaked from 2 to 6 h postinjury. 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. Although thermal injury has been experimentally shown to markedly enhance HMGB1 gene expression in various organs, it required 24 h or more[165]. Proinflammatory cytokines IL-6 and IL-8 increased significantly only from day 2. These findings support the hypotheses that HMGB1 measured on admission has been released from damaged cells and not because of inflammation. Hypovolemia and tissue hypoxia after burn trauma may additionally contribute to the elevation of plasma HMGB1 concentration. Our results may indirectly support the experimental findings of Zhang et al.[128] that HMGB1 released after major burns is associated with the development of immune suppression, and HMGB1 also acts as an immunosuppressor cytokine complementary to its role in the pathogenesis of septic response. 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[128,130]. 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 our previous paper we published that ITBVI guided fluid therapy was associated with earlier normalization of tissue oxygenisation reflected by higher $ScvO_2$ and lower MODS[52]. The earlier normalization of tissue oxygenisation was achieved by more rapid fluid administration. In this regard recent results together with our previous observations[52] suggest

that HUO guided resuscitation might have been a retarded procedure. We also demonstrated previously that ITBVI guided fluid resuscitation had a moderate beneficial effect on the pro-oxidant state but it did not influence the anti-oxidant parameters[52]. 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. Papp and coworkers could not find an association between improved cardiac function and plasma cytokine levels[166], however, Venet and associates, comparing the hemodynamic profile and IL-6 levels in survivors and non-survivors using the pulmonary arterial catheter found higher IL-6 levels, higher cardiac output (CO) and oxygen delivery in survivors. Unfortunately the levels of other cytokines were not studied[167]. 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[52]. Increased CD11a/b/18 expression as a sign of leukocyte hyper activation can promote neutrophil adherence to endothelium causing microvascular plugging by leukocytes[168] that, along with edema, can lead to inadequate tissue oxygenisation. When comparing survivors to non-survivors significantly higher levels of CD11a were reported[169] in non-survivors without a significant difference in CD11b and CD18 levels. Bucky and associates found a close relation between endotoxin levels and CD11b expression[170]. The use of monoclonal antibody directed to the human leukocyte adherence glycoprotein CD18 to block neutrophil adherence to endothelium and intravascular aggregation in a rabbit model of partial-thickness burn led to less edema-formation, and an eightfold increase in live hair follicles was also observed[171]. Systemic administration of monoclonal antibodies directed to CD11b and 18 maintained the blood flow in the burned wound due to blocking of the extravascular migration of neutrophils[86]. Nwariaku and associates have shown that leukocyte

adhesion is L-selectin dependent and CD18 is responsible for tissue damage[84]. Burn trauma is often associated with lung injury. Granulocyte accumulation in the lungs is related to increased CD11b/CD18 expression[172]. 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. It is broadly expressed on hematopoietic cells and involved in neutrophil migration[93,173], and plays a role in leukocyte trafficking and in peripheral granulocyte homeostasis[174,175]. Its role has not been studied in burn injury, but its neutralization increases the resistance to collagen-induced arthritis in mice[176] and its importance has been proven in the cardio-pulmonary-bypass-related inflammatory response[177]. Markedly enhanced expression of CD97 on polymorphonuclear neutrophil granulocytes, and significantly increased proportion of CD97 positive lymphocytes were found in the joint fluids, as compared to the corresponding peripheral blood samples after intraarticular bleeding[87]. 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[178]. Finnerty and associates found elevated levels of human LPS binding protein in patients suffering from SIRS[152] and elevated levels of CD14 were associated with an increased risk of severe sepsis after burn[173]. In murine burn model, CD14-mediated LPS signaling pathway may play a role in the regulation of NF-κB alternative splicing in the lungs after injury[179]. Pharmacologic inhibition of the CD14 signaling pathway in an animal model protected against burn-related myocardial inflammation and dysfunction[180]. Fangand associates found in an animal model that inhibition of CD14 with the amino-terminal fragment of bactericidal/permeability-increasing protein could reduce the development of multiple organ damage resulting from gut-origin endotoxin translocation[91]. 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[181]. Elevated levels of CD14 were associated with an increased risk of severe sepsis after burn injury[182]. 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. Similarly to our results, Jutkiewicz-Sypniewska and co-workers have found decreased antioxidant capacity, and elevated thiobarbituric acid reactive substances (TBARS) (MDA) level in burned children[183]. Bertin-Maghit and co-workers have also found decreased levels of antioxidants, and elevated TBARS in burned patients[76]. Pintaudi and co-workers have found significantly elevated MDA level on admission following burn injury[184].

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[185]. In rat pulmonary contusion model administration of NAC significantly diminished MDA levels in lung compared to controls. NAC treatment prevented the decrease in lung glutathione and significantly lowered serum isoprostane levels, neutrophil infiltration and cytokine levels in the brocho-alveolar lavage during high tidal volume ventilation[186]. Treatment of rats with NAC significantly elevated the GSH, while decreasing MDA level and MPO activity after burn injury. Ocal and associates suggested that NAC has a crucial cytoprotective role in intestinal mucosal barrier and preventive effects against burn injury-induced bacterial translocation[66]. In a rat two hit trauma model administration of NAC increased tissue GSH levels while decreased MDA levels, but it did not influence survival[187]. In humans NAC administration increased serum GSH in patients undergoing IL-2 induced lymphokine-activated killer cell treatment[188]. Similarly to the above cited experimental and clinical data

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. Curbo and associates provided evidence for a novel redox dependent pathway for regulation of cytokine activity by extracellular reduction of intramolecular disulfides at the cell surface by members of the thioredoxin enzyme family[189]. The anti-inflammatory effect of NAC can be based on regulatory effect of ROS on translocation of transcription factor NF-κB to cell nuclei[190]. Moreover, IL-8 production is regulated by mitogen-activated protein kinases, but ROS can alter IL-8 production too. In human gastric carcinoma cells NAC could decrease IL-8 production[191].

In the acute phase of burn injury monocytes are main sources of circulating interleukins. Toumpanikis and associates found that antioxidant supplementation with NAC decreased interleukin production of monocytes at rest and exercise in humans[192]. Radomska-Lesniewska and associates concluded that NAC is an effective inhibitor of TNFα, IL-1β and IL-8 release in endothelial and epithelial cells[193]. NAC administration could decrease tissue infiltration of neutrophils in the lungs, ileum and colon in a rat acute pancreatitis model as well as IL-6 levels[194]. In porcine ischemia-reperfusion model administration of NAC could decrease the inflammatory cytokine and TBARS levels in broncho-alveolar lavage[195]. 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[175].

Among β2 integrins granulocyte CD11a levels were significantly lower in the NAC group from day 4 following injury, similarly to CD18. Nakae et al. found significantly higher levels of CD11a in non-survivors[168] without any significant difference in CD11b and CD18 levels. Our data, together with others are in contrast to the observation of Rodenberg and associates. They found that patients after burn injury were not able to express CD11/CD18 on the same degree as healthy volunteers[196]. The above-mentioned data suggest that 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[197]. It has been shown to be involved in lymphocyte, eosinophil, and monocyte adhesion and emigration. Its role in burn has not been investigated

yet. Higher levels of CD49d can be found in dialysis patients[198]. Regular aerobic exercise seems to protect against vascular disease and it is associated with lower expression of CD49d[199]. Moreover, its levels are increased in patients with preeclampsia[200]. In primary Sjogren's syndrome, which is associated with lymphocytic infiltration in exocrine glands, elevated CD49d expression of lymphocytes was observed[201]. 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. Our data are in concordance with the paper of Puig-Kröger and associates, who found that CD49d expression was dependent on cell maturation, and its induction was abrogated by NAC, which inhibits NF-κB activation and the functional and phenotypic maturation of monocyte-derived human dendritic cells[202].

CD97 is a member of the G protein-coupled receptor[203]. 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. The above-mentioned literatures data may suggest a beneficial effect of lower CD97 expression in NAC treated group.

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[134]. In human studies NAC treatment did not influence the survival147,[204]. 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 sepsis and mortality. Moreover, HMGB1 level was significantly higher in our study both in septic and nonsurvivor patients than in nonseptic and survivor ones on admission; and despite the small study population, it had a predictive value.

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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10. 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**
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3. Csontos C, **Foldi V**, Pálinkas L, Bogar L, Röth E, Weber G, Lantos J. Time course of pro- and anti-inflammatory cytokine levels in patients with burns-prognostic value of interleukin-10. Burns 2010; 36: 483-94. **No. of citation: 1; IF: 1.718**
4. Lantos J, **Földi V**, Roth E, Wéber G, Bogár L, Csontos C. Burn trauma induces early HMGB1 release in patients: its correlation with cytokines. Shock 2010; 33: 562-7. **No. of citation: 3; IF: 3.203**
5. **Foldi V**, Csontos C, Bogar L, Roth E, Lantos J. Effects of fluid resuscitation methods on burn trauma-induced oxidative stress. J Burn Care Res 2009; 30: 957-66. **No. of citation: 1; IF: 1.617**
6. **Földi V.**, Csontos Cs., Major K., Bogár L., Wéber Gy., Rőth E., Lantos J.. Oxidatív stressz és antioxidáns terápia égési trauma után - Az N-acetilcisztein szerepe a klinikai gyakorlatban - Pilot study. Érbetegségek 2009; 2: 35-44.
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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**
2. Csontos C, **Foldi V**, Fischer T, Bogar L. Factors affecting fluid requirement on the first day after severe burn trauma. ANZ J Surg 2007; 77: 745-8. **No. of citation: 10; IF: 0.998**
3. Csontos C, **Foldi V**, Fischer T, Bogar L. Arterial thermodilution in burn patients suggests a more rapid fluid administration during early resuscitation. Acta Anaesthesiol Scand 2008; 52: 742-9.

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4. Csontos Cs, **Földi V**, Fischer T, Bogár L. Mely faktorok befolyásolják az égett betegek folyadék igényét a sérülés utáni első 24 órában? Aneszteziológia és Intenzív Terápia 2007; 37: 68-73.

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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10. Lantos J, Rőth E, Wéber G, **Földi V**, Csontos C. Burn trauma induces early high mobility group box protein 1 (HMGB1) release in patients. British Journal of Surgery 2009; 96(S5): 61. **IF: 4.077**
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Cumulative IF of abstracts related to the thesis: 38.138

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1. Lantos J, Drenkovics L, Rézmán B, **Földi V**, Roth E, Bogár L, Wéber Gy, Csontos Cs. N-acetyl cisztein kiegészítő kezelés hatása a big-endotelin szintre égésbetegségben. Érbetegségek 2011; 18(S1): 15-16.
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This thesis is based on the following presentations

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. Magyar Aneszteziológiai és Intenzív Terápiás Társaság 38. Kongresszusa. 2010. május 13–15., Eger.
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This thesis is based on the following posters

1. Lantos J, **Földi V**, Rézmán B, Drenkovics L, Bogár L, Rőth E, Wéber Gy, Csontos Cs. Sejtfelszíni markerek kórjelző értékének vizsgálata égési sérülést követően. A Magyar Élettani Társaság (MÉT) LXXIV. Vándorgyűlése, és a Magyar Kísérletes és Klinikai Farmakológiai Társaság (MFT) II. közös tudományos konferenciája. 2010. június 16-18., Szeged.
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