Function of granulocytes after burns and trauma, associations with pulmonary vascular permeability, acute respiratory distress syndrome, and immunomodulation

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“Physicians think they do a lot for a patient when they give his disease a name.”

Immanuel Kant
Abstract

A minor physical injury may pose a threat to an organism, but our immune system is set to deal with such threats. If it is functioning properly the effector cells of the immune system will seek out the threat and prevent infection. These cells use an array of weapons to reach their goal, and the weapons may cause collateral damage. In the case of a relatively small injury the organism will cope with this.

Severe physical injury may be an immediate threat to an organism, a threat that often proved deadly throughout evolution. Such injuries may induce massive collateral damage. Nowadays we can initiate advanced critical care for affected patients and save them from imminent trauma-related death. We are therefore faced with the fact that the collateral damage from the immune system may pose a major threat to the patient, the pathophysiology of which is not amenable to direct medical treatment and which leaves us with only passive supportive measures.

For the purpose of this thesis we investigated the role of leucocytes under such circumstances, with specific attention to granulocytes, their release of heparin binding protein (HBP), and the consequences for the development of the respiratory failure that is often classified as acute respiratory distress syndrome (ARDS).

Our main aim was to understand better the role of leucocytes in the development of increased vascular permeability after burns and trauma. In the context of the vasculature of the lungs, this applies to the impairment of oxygenation and the development of ARDS.

More specifically we investigated the impact of trauma on the function of leucocytes such as the dynamic change of certain cell-surface receptors on the leucocytes and in their numbers and immature forms. We wanted to find out if the increased pulmonary vascular permeability after a burn could be mediated through HBP, and whether HBP could be used as a biomarker for respiratory failure after trauma. We also wanted to confirm the possible role of histamine as a mediator of the systemic increase in vascular permeability after burns.

The dynamic change of cell-surface receptors was measured by flow-acquired cytometer scanning (FACS) on blood samples taken after burns. The concentrations of HBP after a burn and other physical trauma were analysed in plasma. Pulmonary vascular permeability after a burn was assessed using transpulmonary thermodilution. The histamine turnover after a burn was
assessed with high performance liquid chromatography (HPLC) for concentrations of histamine and methylhistamine in urine. We confirmed results from earlier investigations that showed altered expression of receptors on leucocytes after a burn, receptors that are intimately associated with leucocyte function in acute inflammation. In a pilot study of 10 patients we measured concentrations of HBP and found them to be increased soon after a burn. This finding was not confirmed in a larger, more extensive and specific study of 20 patients. We did, however, find an association between alterations in the number of leucocytes soon after a burn and pulmonary vascular permeability, indicating that they had a role in this process.

In another study of trauma (non burn) we found an association between the concentration of HBP in samples of plasma taken soon after injury and the development of ARDS, which indicates that granulocytes and HBP have a role in its aetiology. We found a small increase in urinary histamine and normal urinary methylhistamine concentrations but had anticipated a distinct increase followed by a decrease after reading the current papers on the subject. This indicates that the role of histamine as a mediator of increased vascular permeability after burns may have been exaggerated.

We conclude that leucocytes, and granulocytes in particular, are affected by burns and trauma, and it is likely that they contribute to the development of respiratory failure and ARDS. HBP is a candidate biomarker for the early detection of ARDS after trauma, and the white blood count (WBC) is a useful biomarker for the detection of decreased oxygenation soon after a burn.

**Key words:** ARDS, azurocidin, burn, CAP-37, critical care, granulocyte, HBP, histamine, intensive care, leucocyte, leukocyte, mediator, methylhistamine, MOF, oedema, neutrophil, permeability, PMN, trauma, vascular permeability.
List of publications
This thesis is based on the following papers and manuscripts, which are referred to in the text by the corresponding roman numerals.


Other publications to which Joakim Johansson contributed and are not included in the thesis are:


Abbreviations

ANOVA Analysis of variance
APACHE Acute physiology and chronic health evaluation
ARDS Acute respiratory distress syndrome
AUC Area under the curve
BAL Bronchoalveolar lavage
CAP-37 Cationic antimicrobial protein of 37 kD (another name for HBP)
CARS Compensatory anti-inflammatory response syndrome
CD Cluster of differentiation: a system of classification used for cell surface receptors expressed on cells
CPAP Continuous positive airway pressure
CR3 Complement receptor 3 (another name for CD11b)
CRP C-reactive protein
EDTA Ethylenediaminetetraacetic acid
EVLW Extravascular lung water
FTB% Full thickness burn (%)
FiO2 Fraction of inspired O2
GEDV Global end diastolic volume
HBP Heparin binding protein
HES Hydroxyethylstarch
HPLC High performance liquid chromatography
ICU Intensive care unit
ISS Injury severity score
ITBV Intrathoracic blood volume
ITTV Intrathoracic thermal volume
Jv The net outward fluid flux over a membrane (endothelial layer) (cm³·s⁻¹)
LAEDV Left atrial end diastolic volume
Lp The hydraulic permeability of a membrane (endothelial layer) (cm·s⁻¹·cmH₂O⁻¹)
LVEDV Left ventricle end diastolic volume
MFI Mean fluorescence intensity
MODS Multiple organ dysfunction syndrome
MOF Multiple organ failure
PaO2 Partial pressure of oxygen in arterial blood (measured in kPa)
PaCO2 Partial pressure of carbon dioxide in arterial blood (measured in kPa)
\(P_{\text{cap}}\)  The hydrostatic pressures inside the capillary (cmH\(_2\)O)

\(P_{\text{if}}\)  The hydrostatic pressures outside the capillary (cmH\(_2\)O)

PAOP  Pulmonary artery occlusion pressure

PBV  Pulmonary blood volume

PCT  Procalcitonin

PEEP  Positive end expiratory pressure

PMN  Polymorphonuclear leucocyte

PTB\%  Partial thickness burn (\%)

PVPI  Pulmonary vascular permeability index

RAEDV  Right atrial end diastolic volume

ROC  Receiver-operating characteristic curve

RVEDV  Right ventricle end diastolic volume

\(S\)  The surface of the membrane (endothelial layer) (cm\(^2\))

SIRS  Systemic inflammatory response syndrome

SOFA  Sequential organ failure assessment score

TBSA (\%)  Total burn surface area (\%)

TLR-4  Toll-like receptor-4

WBC  White blood cell count

VAP  Ventilator associated pneumonia
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I Introduction

1.1 Trauma
A severe injury is often life-altering, and has widespread effects on the patient’s future. Many epidemiological studies have confirmed that it contributes to morbidity and mortality in the general population. As many as 80% of deaths from trauma today are at the scene [1]. Among the patients who survive to be admitted to hospital organ failure is a feared complication, though its incidence is decreasing [2]. There is no specific treatment other than supportive measures, and it also contributes to morbidity and mortality [3]. Although the age of injured patients is increasing [4] the condition is still most common among young people [5], and among men more than women [4-5]. It has often been stated that death after trauma occurs within three different time-frames, and indeed Soreide et al. confirmed a weak trimodal temporal distribution that also mirrored the different causes of death [4]. Some patients die early, often at the scene of the injury, and often from exsanguination or severe damage to the central nervous system (CNS). Some die in hospital within 48 hours for various reasons, and some die within a longer time frame and often from organ failure secondary to the primary injury. This thesis deals with organ failure.

Whether the injury involves a burn or not, all the studies in this thesis start with an event that has serious effects on the patient, whether functional, emotional, social, or immunological. If the patient survives such an event it will be followed by systemic inflammation, which in turn may be followed by organ failure. Somewhere along the way leucocytes are activated, and it is generally thought that they take part in the pathological process of organ dysfunction or failure after burns and trauma [6-8]. The exact mechanism by which leucocytes contribute to acute respiratory distress syndrome (ARDS) is not clear.

You need only have a minor burn to appreciate its capacity to initiate an inflammatory response. Within minutes the skin swells and becomes red, and it is painful, often beating in time with the pulse. This is an immediate and strong response, and one may wonder what happens if more than 20% of the skin is affected by the same reaction. We know that such burns were deadly in the era before modern health care, and patients died in what was then called “burn shock”. Shock is defined as inadequate oxygenation of the tissues. The shock
after a burn resembles hypovolaemic shock, but perhaps the term “intravascular hypovolaemic shock” better describes the state, as total body water is normally not exceptionally low. The problem is that fluid leaves the circulation and enters the extravascular space. The circulatory state of “burn shock” has been described by Tricklebank [9]: “Loss of intravascular volume to the interstitium results in a unique phenomenon called burn shock, which is a combination of distributive, hypovolaemic and cardiogenic shock.”

As this thesis deals with patients with burns, and patients who have had a mechanical trauma, I shall use the term “burns” for burns, “trauma” for mechanical trauma, and “injury” when I mean either or both.

1.2 Inflammation: basic concepts and history

Even though our biological and biochemical knowledge of the process of inflammation is acquired relatively late during the medical history, the symptoms of inflammation were described long ago. Dolor, calor, rubor, tumour (pain, warmth, redness, and swelling) are the symptoms that Celsus originally used to describe inflammation during the first century [10]. They are as valid today as they were then, and listing them offers a pedagogic way of looking at the process. The warmth and redness is explained by vasodilatation, the swelling caused by increased vascular leakage. The pain originates from the fact that certain proinflammatory substances also modulate nerve transmission in free nerve endings.

1.3 Burns, trauma, inflammation, and organ failure

This thesis deals with a specific complication of injury, the secondary organ dysfunction that may follow it. The term multiple organ failure (MOF) is often used, and includes ARDS. It may present soon after the injury, but death from organ failure after trauma often comes later. The systemic inflammatory response syndrome (SIRS) is a condition that may arise from physiological stressors as divergent as, for example, infection, pancreatitis, and ischaemia-reperfusion injury, and it is often seen after injury [11]. The diagnosis of SIRS is based on the presence of two or more of the following criteria:

- **Temperature**: $\leq 36^\circ C$ or $\geq 38^\circ C$
- **Heart rate**: $\geq 90$ beats/minute
- **Respiratory rate**: $\geq 20$ breaths/minute or $\text{PaCO}_2 < 4.0$ kPa
White blood cell count  
≥ 12,000 or ≤ 4,000 cells/mm³
or > 10% bands (immature form)

There is overwhelming evidence that the function of the immune system is altered after trauma [12], and more specifically that the function of leucocytes is altered, in particular granulocytes [13]. This, combined with the fact that large amounts of infiltrating granulocytes are found in the lungs of patients with ARDS, led reviewers to conclude that the issue is not so much whether granulocytes contribute to secondary injury to organs after injury, but rather how they do it [6, 14].

Both trauma and burns induce SIRS, which is part of immunological activation, and there is probably a connection with the later development of ARDS, which is also thought to be dependent on immunological activation [15]. One may therefore look upon the relatively harmless condition of SIRS after injury as a part of the continuum that culminates in ARDS, with a mortality of 40% [2].

1.4 ARDS

1.4.1 Basic concepts

ARDS was first described in 1967 [16]. The key findings listed in the original description were decreased oxygenation that did not respond well to treatment with oxygen, combined with signs of infiltration on chest radiographs. Of the 12 patients studied 7 died, and necropsy showed hyaline membranes, interstitial inflammation, intra-alveolar oedema, and haemorrhage. The optimal definition and treatment of the syndrome is still a matter for debate.

ARDS is a mixture of ventilation-perfusion mismatch and a problem with diffusion, which leads to decreased oxygenation that does not respond well to increased inhalation of oxygen. We use the term fraction of inspired O₂ (FiO₂) to define the fraction of oxygen in the gas that we breathe. The ability of the lung to oxygenate the blood may obviously be measured by the partial pressure of O₂ in the blood (PaO₂). Because we often use increased amounts of inhaled oxygen to overcome problems with decreased PaO₂, we compensate by constructing a ratio with PaO₂ as the numerator and FiO₂ as the denominator. This ratio, PaO₂:FiO₂, is the basis on which we define ARDS. The mismatch of ventilation and perfusion in the lungs can be treated by positive pressure ventilation, which aims to open up the whole lung. The diffusion problem, which is caused by thickening of the interalveolar space and accumulation of alveolar fluid, is as
important and may be overcome with an increase of FiO$_2$ (and with increased ventilator pressures).

Incidences have been reported to be from 1.5-78.9/100,000 person-years but may be considered uninteresting from the clinical point of view as ARDS always follows from another state that requires hospital care [2, 17-19]. The existence of ARDS in practice depends on access to intensive care, because the degree of oxygenation required would often not be compatible with life without a ventilator. Facilities for analysis of blood gases must be at hand to set the diagnosis and grade ARDS correctly.

More interesting, perhaps, is to express the incidence in a population at risk. This is also tricky as it will depend to a large extent on the group studied and on the fact that different definitions of ARDS exist. The reported range here spans from 4%-26% after trauma in general [2]. After burns the incidence reported varies a lot, probably because of the different characteristics of injuries. In a randomised trial of patients with a mean percentage total body surface area (TBSA%) of 35% the prevalence of ARDS was 42% in the total series [20]. In our centre (Burns Intensive Care Unit, Linköping University Hospital) we found an incidence of 56% in a small study (n=16) of burned patients whose TBSA% was 39.6% [21].

During the period in which the American-European Consensus Conference (AECC) criteria were used (1994-2012), it was clear that the incidence declined [2], probably because of wider use of the results from the lower tidal volume study by the ARDS network [22], better treatment for underlying diseases and states, a restrictive transfusion strategy, and treatment protocols for sepsis and ventilator-associated pneumonia (VAP).

Striking features of ARDS are that causal treatment is lacking [23], and that the same syndrome may arise from such different triggering events.

1.4.2. Criteria for ARDS

1.4.2.1 The AECC (American European consensus conference) criteria

The definition most often used from 1994 until 2012 was the result of the AECC in 1994 [24]. This is the definition used in study IV. The AECC criteria for acute lung injury (ALI)/ARDS are: acute onset; bilateral infiltrates on chest radiograph; no evidence of left atrial hypertension, with pulmonary artery occlusion pressure (PAOP) $\leq$18 mmHg if measured; and PaO$_2$: FiO$_2$ ratio $\leq$40 kPa (ALI) and <27 kPa (ARDS).
1.4.2.2 The Berlin criteria

New criteria were published in 2012 after a conference in Berlin - the so-called Berlin criteria [25]. They are: acute onset (within 1 week of known injury); bilateral opacities on chest radiograph (not explained by effusions, collapse, or nodules); and respiratory failure not fully explained by heart failure or fluid overload (objective assessment such as an echocardiogram is recommended if there are no risk factors).

The severity of ARDS is graded as: mild: $40 \geq \text{PaO}_2\text{FiO}_2 > 27$ kPa with PEEP or CPAP $\geq 5$ cm H$_2$O; moderate: $27 \geq \text{PaO}_2\text{FiO}_2 > 14$ kPa with PEEP $\geq 5$ cm H$_2$O; or severe: $14 \geq \text{PaO}_2\text{FiO}_2$ with PEEP $\geq 5$ cm H$_2$O.

1.4.3 Problems encountered with the definition of ARDS

There are inherent problems with the definition and treatment of ARDS that may affect the incidence and obstruct studies of its pathophysiology. One lies in the fact that that the AECC did not require PEEP for the diagnosis but looked only at $\text{PaO}_2\text{FiO}_2$. When arterial blood gases are measured before or immediately after initiation of ventilatory support many patients will fulfill the criteria. When the next sample is drawn, after some hours with ventilator support and in accordance with the recommendations of the ARDS network, the ratio may be much improved and no ARDS present according to the strict definition. This improvement depends a lot on the PEEP applied in the ventilator, allowing for us to decrease the $\text{FiO}_2$ with a preserved $\text{PaO}_2$. The patient is then classified as not having ARDS even though the initial pathophysiological disturbance that caused ARDS may still be present. This problem is partly accounted for with the new Berlin criteria, which require CPAP or PEEP $5$ cm H$_2$O to state that ARDS is present. Because a patient who requires PEEP $18$ cmH$_2$O may be considered more affected by ARDS than a patient who requires PEEP $8$ cmH$_2$O to reach specified targets, a more extensive correction for PEEP may be useful for successful research into ARDS. The Murray lung injury score (LIS) takes PEEP into account to a certain degree, and also includes static compliance of the respiratory system and how widespread the effusions are on the chest radiograph [26].

Another problem with the definition of ARDS has been that signs of congestive heart failure exclude ARDS, but patients with pre-existing congestive failure can
also develop ARDS after injury. They may even be predisposed to it, as it is likely that even a small increase in capillary permeability will result in a capillary leak, depending on baseline increased PAOP. For patients with subclinical congestive failure, one may also apply similar reasoning that can also be problematic for the diagnosis of ARDS. The capillary leak in the lung and the systemic circulation may force the treating physician to resuscitate with large amounts of fluid. This may lead to increased PAOP in a patient bordering on congestive failure. This patient would by definition not have ARDS, but by defining him or her as healthy we are asking for problems when we are studying the pathophysiology of ARDS. The new definition (after the Berlin conference) handles the last issue of increased PAOP well, and states that if the suspected ARDS follows an acute state that is known to cause ARDS, this is enough regardless of known or suspected increased PAOP. If there is no such predisposing state, one must exclude increased left atrial pressure (PAOP is thought to reflect left atrial pressure), for example with echocardiography. There is also the issue of timing, as ARDS may follow various physiological stressors. Earlier ARDS and MOF were thought to be related to sepsis, even when it presented after trauma. This may be the case, but nowadays the generally accepted theory is that ARDS after trauma may be either early and an aseptic reaction to the trauma itself, or late and the result of either the trauma or the following events such as operations, transfusions, hypotension, or sepsis [2, 27].
Table 1. Different characteristics and possible division of ARDS.

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timing</td>
<td>Early</td>
<td>&lt;48-72 hours of initiating event. If trauma, related to the trauma itself, hypovolaemic shock or transfusions.</td>
</tr>
<tr>
<td></td>
<td>Late</td>
<td>&gt;48-72 hours of initiating event. If trauma, often related to secondary infections such as ventilator-associated pneumonia, or aspiration in relation to decreased consciousness.</td>
</tr>
<tr>
<td>Anatomical</td>
<td>Direct</td>
<td>A pulmonary contusion in case of trauma, possibly less dependent on a mediating factor. Possibly an inhalation injury in burns. Pneumonia in the case of infection.</td>
</tr>
<tr>
<td></td>
<td>Indirect</td>
<td>Non-pulmonary trauma in case of trauma, such as burns. Non-pulmonary focus if induced by an infection. A mediating factor must be present.</td>
</tr>
<tr>
<td>Infectious</td>
<td>Sepsis-induced</td>
<td>Related to an infection, most likely severe sepsis or septic shock.</td>
</tr>
<tr>
<td></td>
<td>Not sepsis-induced</td>
<td>Related to another initiating event - for example burns, trauma, pancreatitis, hypovolaemic shock, transfusions.</td>
</tr>
</tbody>
</table>

The problem with research into ARDS is that the research worker wishes to use the entity “ARDS” as a diagnosis, when it is really a syndrome. To make it even more complicated, the syndrome ARDS may be related to many different initiating events, possibly with different characteristics and aetiologies.

1.5 Innate immunity, basic

Our immune system is divided into the adaptive system, which deals with infective agents that have been encountered before and which takes some time to start up, and the innate system, which has an innate ability to recognise agents or infections as foreign or dangerous and may start immediately. We know today that the two systems overlap and interact to some extent. This thesis deals only with the innate immune response.

Leucocytes are the effector cells of the immune system, most of which are circulating in the blood. There are many different types and subtypes, all of which are generated in the bone marrow from haematopoietic stem cells that divide and differentiate into the different types [28]. Monocytes and lymphocytes are also present in the circulation but this thesis will focus on the role of granulocytes.
1.5.1 The granulocyte (polymorphonuclear leucocyte, PMN)

In healthy humans the most abundant circulating leucocytes (40%-70%) are the granulocytes (sometimes also named polymorphonuclear leucocytes, PMN). They stem from the myeloid line of differentiation and are further subdivided into neutrophils, eosinophils, and basophils, of which neutrophils are the most abundant. The normal maturation process for granulocytes takes 2 weeks in the bone marrow, during which the cell differentiates from myeloblast to myelocyte and then to the mature granulocyte with the characteristic segmented nucleus.

![Diagram of granulocyte maturation](image)

Fig. 1. The maturation of granulocytes in the bone marrow. From [28], with kind permission from Springer Science and Business Media.

The granulocyte normally survives 12-14 hours in the circulation when it does not encounter bacteria or become activated in any other way. We do not know how long a leucocyte survives after burns or trauma. In case of an infection, the granulocyte will roll and adhere to the inner side of the endothelium and then transmigrate to the extravascular space. Briefly, its next step to reaching the infective site is chemotaxis towards the gradient of chemotactic stimuli, and when it reaches a microbe it fulfils its mission and kills it. Granulocytes are called granulocytes simply because they contain a lot of granules. There are four different types of granules: secretory vesicles and tertiary, secondary, and primary granules [29-30]. The order in which granules are formed during the maturation process and their position in the granulocyte cytoplasm are keys to when and where these granules are released, the so called “targeting by timing” theory [29-30].
1.5.2 Secretory vesicles

The secretory vesicles were first described as late as in 1987, and are the last to form in the maturation process. They may even be formed after the granulocyte has been released from the bone marrow, and are the most easily mobilised after activation [31]. The mobilisation of the granule takes place as an exocytosis, which upregulates proteins originally attached to the inside of the granule’s wall and moves them on to the outer surface of the cell [32]. The other obvious result of exocytosis is that what was contained in solution in the vesicle is released into the surroundings.

Among other receptors the secretory vesicle wall contains CD11b, CD14, CD16 [33] and probably proteinase 3, [34] which are upregulated upon exocytosis. Tapper et al. have shown that secretory vesicles also contain heparin binding protein (HBP) in solution [35]. CD11b is a receptor that is used for activation of granulocytes in case of firm adhesion to the endothelial cells, which is a prerequisite for extravasation [36]. CD11b is also sometimes called Mac-1, CR3 or $\alpha_m\beta_2$. CD16 is also called Fc$\gamma$RIII and is a receptor used by granulocytes to bind to the constant part (the Fc-part) of an antibody during the process of phagocytosis. CD14 can briefly be described as a coreceptor of toll-like receptor 4 (TLR-4), which is a pattern recognition receptor that binds to lipopolysaccharide (LPS), and mediates some of the symptoms seen in Gram-negative sepsis. The exocytosis of secretory vesicles, which upregulates receptors on granulocytes, transfers the former inactive circulating granulocyte into an activated state, where it becomes capable of interacting with its surroundings. This is a prerequisite for further tasks of granulocytes such as transmigration, chemotaxis, oxidative burst, and phagocytosis.

1.5.3 Tertiary, secondary, and primary granules

These granules contain many different proteins, both in solution and attached to the membrane of the granule [33]. Stronger stimulation of the granulocyte is needed in vitro to release these granules, an observation that fits with their formation earlier in the granulocyte’s maturation process and released in the later stages of its activation in vivo [30], the “targeting by timing” theory. There are different ways of classifying these granules based on their content of, for example, gelatinase.
1.5.4 Brief description of the multiple steps of granulocyte extravasation

Granulocytes circulate in the blood in a non-activated state. In the case of localised proinflammatory stimuli they will interact with the endothelial cells and start a reaction, the aim of which is to leave the blood vessel and save us from a potential infection. The process has been reviewed by Ley [36]. The first part of this reaction is rolling of the granulocyte alongside the endothelial layer, mediated by weak interactions of endothelial cell surface receptors (e.g. P- and E-selectin) with carbohydrate structures on the granulocyte (e.g. P-Selectin Glycoprotein Ligand-1; PSGL-1) [36]. Rolling is a prerequisite for subsequent firm attachment of the granulocyte to the endothelium. Granulocyte firm adhesion is mediated predominantly by members of the β2 integrin family (CD11a/CD18; LFA-1 and CD11b/CD18; Mac-1) interacting with receptors on the endothelial cells belonging to the immunoglobulin superfamily (e.g. ICAM-1). The next step in the extravasation cascade consists of transmigration across the vessel wall, often in between two endothelial cells, a process not as thoroughly studied and probably involving several junctional adhesion molecules [36].

Fig. 2. Diagram of neutrophil adhesion and transendothelial migration. In response to inflammatory stimuli, adhesion molecules such as selectins are upregulated on endothelial cells, and granulocytes roll along the vascular endothelial wall through selectin-mediated weak interactions. This is followed by firm adhesion of granulocytes to endothelium through binding of integrins on the granulocyte surface to the endothelial cell surface. Subsequently, granulocytes transmigrate through the microvascular endothelium by a process involving complex interactions with endothelial cell-cell junction molecules. CD11b is Mac-1. Reprinted from Neutrophil transmigration, focal adhesion kinase
1.6 Vascular permeability in general

1.6.1 Basic concepts
The exchange of fluid over endothelium in a vascular bed was described already in 1896 by Starling [38] and defines the flux of fluid $J_v$ through a membrane (an epithelium) as follows:

$$J_v = S \times L_p \times (P_{cap} - P_{if}) - \sigma \times (\pi_{cap} - \pi_{if})$$

$J_v$ is the net outward fluid flux ($cm^3/s$). $S$ is the surface of the membrane ($cm^2$) and $L_p$ is the hydraulic permeability of the surface ($cm/s/cmH_2O$). These two entities are properties of the membrane (in this case the endothelial layer). $P_{cap}$ and $P_{if}$ ($cmH_2O$) are the hydrostatic pressures inside and outside of the vessel. Because $P_{cap}$ is larger than $P_{if}$, the difference $P_{cap} - P_{if}$ must in all cases be a positive number (except in the case of total occlusion because of compartment syndrome, for example a total cerebral infarction). This corresponds to a net filtration of fluid from the vessel and is partially counteracted by the factor $-\sigma$ ($\pi_{cap} - \pi_{if}$). $\pi_{cap} - \pi_{if}$ is the difference between oncotic pressures inside and outside of the vessel. Because $\pi_{cap}$ is normally larger than $\pi_{if}$, the difference $\pi_{cap} - \pi_{if}$ is normally positive, and the factor in the Starling equation above $-\sigma$ ($\pi_{cap} - \pi_{if}$) corresponds to a net reuptake of fluid to the vessel. $\sigma$ is the reflection coefficient. $\sigma$ is a number between 0 and 1 and it indicates the permeability for large molecules. If it is set to 1 the reuptake of fluids is maximal, which would be the case if the vessel wall did not allow any flux of large molecules at all and the oncotic pressure difference across the vessel wall could maximise its effect.
In a healthy patient this is almost the case, and $\sigma$ is close to 1. This thesis deals with states of increased permeability of vessels. The problem may be rewritten as the problem of decreased $\sigma$ but I shall not deal with explicit numbers of $\sigma$. If $\sigma$ was set to 0 it would correspond to large molecules moving freely across the membrane and hence no oncotic force for reuptake of fluid to the vessel. During normal conditions the filtration is somewhat larger than the reuptake of fluids. The lymphatic system accounts for the transportation of excess fluid from the tissues back to the circulation. In cases of increased vascular permeability, the lymphatic system may increase its capacity. If the capacity of the lymphatic system to transport excess fluid back to the circulation is exceeded, oedema will result. Lymph from the lower part of the body is transported through the thorax via the thoracic duct, and increased pressure in the thorax (PEEP) will have an adverse effect on the capacity of the lymphatic system.

1.6.2 Vascular permeability and burns

A burn induces regional inflammation with locally increased vascular permeability. This is a problem, but I will not focus on local increases in vascular permeability or on local formation of oedema in burned tissue. Systemic vascular permeability is always increased after a severe burn; the limit is often set to 20% TBSA for systemic effects but this is an arbitrary limit. Textbooks and review papers often mention histamine, serotonin, and oxidative radicals as likely mediators of this systemic reaction [39-41].

According to the principle for exchange of fluids over an endothelial lining described above, there are factors other than vascular permeability that affect the amount of fluid extravasated. The actual physiological changes in the interstitial space are not easy to study in vivo, but in 1989 it was shown in an ex vivo preparation model of rat skin that the so-called imbibition pressure was dramatically increased immediately in the burned skin after a burn. The imbibition pressure is the combined forces of the hydrostatic pressure, $P_{if}$, and the colloid osmotic pressure, $\pi_{if}$, in the extravascular space of burned skin [42], which drives the increased net outward fluid flux after a burn. Although interesting, these studies are hampered by the fact that they cannot account for the changes that follow a burn such as inflammation and resuscitation. The negative imbibitions pressure was also measured in vivo in rats by Shimizu et al. [43]. They found early considerable negative imbibition pressure in a deep burn that returned to normal after 50 minutes. In a superficial burn they found no large change in the imbibitions pressure but, somewhat unexpectedly, more oedema than in the deep burn. The explanation may be that the deep burn
induces more degradation of extravascular tissue proteins such as collagen (the proposed mechanism of the strong negative imbibition pressure [44]), but that the coagulation of tissues does not permit any circulation in the burned skin and so less oedema will follow. Because the imbibition pressure was not affected in the superficial burn but even more pronounced oedema was detected, the conclusion of Shimizu et al. was that the oedema in superficial burns was mediated by increased vascular permeability, presumably but not proven so, by an increase in oxygen radicals [43]. Because rats were resuscitated in this study, it resembles clinical conditions better than the ex vivo preparations of Lund et al., but it is still hampered by the possible interspecies differences between rats and humans and it does not specifically address the issue of the increase in systemic vascular permeability.

Clinical studies of oedema after burns are relatively few, some are hampered by problems of methods, and some are old. One takes aim at the possible action of oxidative radicals [45]. Here Tanaka et al. showed that giving ascorbic acid reduced the resuscitation volume needed to reach specific targets, and decreased weight gain and respiratory dysfunction after burns, presumably by counteracting oxidative damage. Vlachou et al. compared the effect of part-colloid (hydroxyethyl starches, HES 6%) resuscitation with that by crystalloid only in a prospective randomised trial [46]. They found that part-colloid resuscitation reduced the increase in C-reactive protein (CRP) and weight gain, which reflected less oedema. There is also a randomised study by Belba et al. that compared hypertonic resuscitation with a standard protocol [47]. In the early phase more fluids were given to the hypertonic group, but in the end there was a trend towards less fluid in total being given to it. Goodwin et al. studied the difference between albumin 2.5 % and standard (crystalloid) resuscitation guided with the help of a pulmonary artery catheter and found that less fluids were given early in the albumin group, but later the extravascular lung water in this group increased [48]. The interpretation was that albumin, although beneficial early, extravasated and interfered with the restoration of fluid balance in the lung later. In another randomised trial, Kravitz et al. investigated the effect of plasmapheresis, but found that it had no effect other than to reach the endpoints in the treatment group faster [49]. The results of these randomised clinical trials are summarised in Table 2.

None of the five randomised trials showed any differences in hard endpoints such as mortality. The ascorbic acid study came closest with less degree of
respiratory failure. None of the studies resulted in any major changes in the early resuscitation of burn patients.

The hallmark of clinical research into burn resuscitation is still the work of Baxter and Shires from 1968 [50], which resulted in the so-called Parkland formula for burn resuscitation, named after the hospital where the work was done. It postulated that the fluid requirement (ml) for acute burn resuscitation is $\text{TBSA}\% \cdot \text{weight (kg)} \cdot 4$. Simple arithmetic shows that the volume that is needed is large if the burn is large. An 80 kg patient with a 50% burn, for example, should have 16000 ml. This amount is supposed to be divided into halves, the first half given within 8 hours of the burn and the second during the next 16 of the first 24 hours.

Endorf and Dries recently reviewed the topic of acute burn resuscitation [51]. There are many case-control trials that have addressed the issue of increased vascular permeability, formation of oedema, and weight gain, but naturally they have had no major impact on clinical decision-making because bias is likely. It is obvious to any clinician who treats burns that patients gain a tremendous amount of weight during the early phase, and the generalised oedema is often impressive. This phase is then followed by a polyuric phase where fluid balance is restored.

Table 2. Randomised trials that addressed the issue of oedema after burn injury.

<table>
<thead>
<tr>
<th>Author (Year)</th>
<th>TBSA(%)</th>
<th>FTB(%)</th>
<th>No</th>
<th>Intervention</th>
<th>Results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanaka et al. (2000)</td>
<td>63</td>
<td>53</td>
<td>51</td>
<td>40</td>
<td>37</td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>Vlachou et al. (2010)</td>
<td>23.5</td>
<td>32.5</td>
<td>9</td>
<td>18</td>
<td>26</td>
<td>Part HES 6% compared with crystalloid only</td>
</tr>
<tr>
<td>Belba et al. (2009)</td>
<td>23.5</td>
<td>32.5</td>
<td>9</td>
<td>18.5</td>
<td>110</td>
<td>Hypertonic compared with crystalloid</td>
</tr>
<tr>
<td>Goodwin et al. (1983)</td>
<td>53</td>
<td>48</td>
<td>?</td>
<td>?</td>
<td>79</td>
<td>Albumin 2.5% compared with crystalloid</td>
</tr>
<tr>
<td>Kravitz et al. (1989)</td>
<td>49.4</td>
<td>52.3</td>
<td>37.3</td>
<td>24.6</td>
<td>22</td>
<td>Plasmapheresis</td>
</tr>
</tbody>
</table>

CRP C-Reactive Protein, TBSA% percent total body surface area burned, FTB% percent full thickness burn.

In a review by Keck et al [41] it is stated that better understanding of how increased vascular permeability after burns is mediated from the burn to the systemic circulation is of “considerable clinical importance”.
1.6.3 Vascular permeability and ARDS

ARDS is characterised by definition as having a decreased oxygenation ratio, the so called P:F ratio (PaO₂:FiO₂). It is generally thought that there is increased vascular permeability in the lungs in ARDS, and that this is the reason for the fluid retention that results in the reduced capacity for oxygen diffusion [6, 52-53].

The pulmonary leakage of protein-rich fluid from the blood stream is a hallmark of ARDS, but the biological mechanism responsible for it has not been elucidated, though reviewers have suggested that granulocytes are responsible [6, 8]. The finding of large quantities of leucocytes in histological studies and from bronchoalveolar lavage (BAL), combined with the known ability of these cells to degrade tissue, have lead to the conclusion that leucocytes may have an important role in the development of ARDS [8, 54-56].

Fig. 3. Changes evident in a lung affected by ARDS (right) compared with a healthy lung (left). Note specifically the gap junctions in between endothelial cells, adherent granulocytes, extravasating granulocytes, granulocytes present in the alveolus, and the oedematous fluid that fills the alveolus. [52]

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1.7 Mediators of vascular permeability

1.7.1 Histamine

Histamine is a classic mediator of inflammation, most known for its role in anaphylactic reactions [57]. It has also been suggested by textbooks and reviews that it is one of the most important mediators of increased vascular permeability after burns [39-40]. The evidence for this statement is mostly from animal studies [58-59], and from a few human studies that have given divergent results [60-61].

Findings from animal models may not apply to human physiology for different reasons. Firstly, a model may not take account of all the clinical factors. Secondly, there may be inherent differences between human and animal physiology, in this case the immune system. For example, are there interspecies differences in subpopulations of mast cells [62].

Papp et al. investigated the role of histamine in a pig model of burns [63], and found increased local concentrations in burned tissue, which suggests that it may have a role as a mediator of locally increased vascular permeability. The systemic concentration of histamine was initially moderately increased and then returned to the reference range. The current knowledge about the role of histamines in human burns is by modern standards scarce.

1.7.2 Heparin binding protein (HBP)

1.7.2.1 Experimental studies on HBP

Animal and in vitro experimental studies have shown that granulocytes can increase vascular permeability without simultaneous tissue destruction [64] and that they do so when adhering to the endothelial layer. We have known for about 10 years that this effect is mediated, at least in part, by a neutrophil protein called HPB, which is secreted from secretory vesicles at the time of granulocyte adhesion. It is also known as azurocidin and cationic antimicrobial protein of 37 kD (CAP-37)) [35, 65-66].

The protein was first identified in 1984 by Shafer et al. [67]. Later, other groups independently of each other also identified it, which is why it has three different names. Later it was discovered that the molecular weight is actually 29 kD. I will refer to it as HBP. It belongs to the serine protease superfamily, and structurally has many similarities with other serine proteases. The primary and
three-dimensional structure has 80% homology with that of elastase, another granulocyte granule compound [68-69]. HBP is, however, devoid of catalytic activity because of two mutations in the otherwise well-conserved catalytic triad that is characteristic of all serine proteases. When a granulocyte adheres firmly to the endothelial layer by interaction of its integrin receptors (CD11b) with the countereceptor on the endothelial cell (intercellular adhesion molecule-1, ICAM-1), it is probably a signal for it to release secretory vesicles [35]. As secretory vesicles contain HBP, it will be deposited in close proximity to the endothelial cell and may even be trapped inside a small compartment that has developed under the adherent granulocyte [70]. The possible concentration of HPB in such a compartment may be much higher than in free-flowing conditions. It has a strong positive charge [69], which creates an affinity for the endothelial cell membrane where the protein accumulates and is left by the transmigrating granulocyte. No specific receptor is identified for HBP, but pretreatment of the endothelial cells with heparinase and chondroitinase decreases the binding of HBP on endothelial cells, which suggests that the negatively-charged surfaces of the glycocalyx act as binding sites for HBP [71]. Originally the antimicrobial properties of the protein were given the most attention, but later it became clear that it had other effects of importance for acute inflammation such as the mediation of increased vascular permeability [65], arrest of - and regulation of - cytotoxic effects in monocytes [71-72], and endothelial cell upregulation of E-selectin and ICAM-1 (which are important for granulocyte rolling and adhesion [73]). Of interest when the possible role of HBP in the development of MOF is examined is that one of the compounds known to inhibit its action is aprotinin (Trasylol®) [65]. The same drug was shown to decrease trauma-induced MOF in a two-hit model in sheep, and the results indicated that the effect was mediated by the altered function of granulocytes [74].
1.7.2.2 Clinical studies of HBP

Linder et al. studied the possible predictive value of measuring plasma HBP in adult patients who presented to the emergency reception in Lund with fever. Such plasma samples were saved in 233 cases and later HBP concentration was analysed. The results showed that HBP had a better sensitivity and specificity than interleukin 6 (IL-6), white blood cell count (WBC), C-reactive protein (CRP), lactate and procalcitonin (PCT), when used to predict whether or not the patient would proceed to develop severe sepsis or septic shock [75]. The same authors recently showed that the concentration of HBP correlated with the severity of disease and mortality in a cohort of mixed patients in ICU [76]. The discriminating ability between sepsis and no sepsis was not as strong in this cohort as in the former study, probably because the patients were sicker and a larger variety of diagnoses was included (including surgical patients).

Chew et al. recently showed in a similar study that plasma HBP measured early in the course of the disease was above normal in patients with shock in the ICU but no higher in patients with septic shock than patients with other types of shock [77]. Recently, Llewelyn et al. investigated the discriminatory power of a set of biomarkers, of which HBP was one, to diagnose sepsis in a mixed ICU [78]. The result was in accordance with that of Chew et al, in that HBP has no
such discriminatory power when the group being tested is ill enough to require intensive care.
Kaukonen et al. have recently analysed concentrations of HBP in plasma from patients with confirmed influenza H1N1, and found higher concentrations in patients who were dependent on mechanical ventilation but not higher in (the few) patients with severe sepsis or septic shock [79]. The same authors also analysed plasma concentrations of HBP in 59 patients included in a prospective, randomised trial that was designed to test differences in outcome when patients with influenza H1N1 was treated with the granulocyte colony-stimulating factor analogue Filgrastim® [80]. The result was that concentrations of HBP were increased in the Filgrastim® group on day 4 of intensive care but not on day 7.
From all the later clinical studies, it is apparent that the cut-off point of 15 ng/ml, which was proposed by Linder et al in their first study, was far too low when the test was used in intensive care.
A case series of three patients has also been described, in which resolution of the circulatory instability was paralleled by normalisation of concentrations of HBP [81].
The clinical studies of HBP in intensive care are summarised in Table 3.
Table 3. Exploratory studies of plasma HBP concentrations with relevance to intensive care.

<table>
<thead>
<tr>
<th>First author</th>
<th>No.</th>
<th>Type of study</th>
<th>Inclusion</th>
<th>Primary findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linder et al. 2009</td>
<td>233</td>
<td>Observation</td>
<td>Medical patients at emergency reception with a fever</td>
<td>Excellent discriminatory ability of HBP to sort out patients later developing severe sepsis/septic shock. Not followed over time.</td>
</tr>
<tr>
<td>Beran et al. 2010</td>
<td>3</td>
<td>Case reports</td>
<td>ICU</td>
<td>High initial HBP, association in time of resolution of organ failure with normalisation of HBP</td>
</tr>
<tr>
<td>Chew et al. 2012</td>
<td>53</td>
<td>Observation</td>
<td>Septic and non-septic shock</td>
<td>High levels in all patients regardless of shock origin. Not followed over time.</td>
</tr>
<tr>
<td>Linder et al. 2012</td>
<td>179</td>
<td>Observation</td>
<td>151 severe sepsis or septic shock, 28 non-septic critical conditions</td>
<td>High levels in all patients, even higher in patients with sepsis. Association of levels of disease severity and mortality. Association of non-HBP normalisation with mortality.</td>
</tr>
<tr>
<td>Kaukonen et al. 2013</td>
<td>29</td>
<td>Observation</td>
<td>Patients in intensive care unit with confirmed H1N1-influenza</td>
<td>High concentrations in all patients, even higher in those with low oxygenation index and those with mechanical ventilation. Not higher in patients with severe sepsis/septic shock.</td>
</tr>
<tr>
<td>Kaukonen et al. 2013</td>
<td>59</td>
<td>Intervention</td>
<td>ICU, randomisation to Filgrastim® or placebo</td>
<td>Higher plasma HBP concentrations in treatment group on day 4 but not day 7. No correlation to oxygenation failure.</td>
</tr>
<tr>
<td>Llewelyn et al. 2013</td>
<td>219</td>
<td>Observation</td>
<td>Mixed diagnoses in high dependency and intensive care unit</td>
<td>No discriminatory power of HBP to sort out patients with sepsis from non-septic</td>
</tr>
</tbody>
</table>

1.8 Research approach to study ARDS or MOF

When ARDS and MOF are studied in animal or human models, the choice has to be made about which model best induces the state that is supposed to be studied and resembles the clinical conditions best. There has been many attempts to modulate the course of organ failure in sepsis, some of which have proved promising in the laboratory but clinically failed completely [82]. The recent withdrawal of activated protein C (APC, Xigris®) from the market is symptomatic of the inherent difficulties of treating sepsis-induced organ failure.
Sepsis, severe sepsis, and septic shock are, although rather clearly defined, syndromes that may result from many initiating events. For example, the microbe may be Gram positive, Gram negative, aerobic, anaerobic, and so on, and the focus of infection may be pulmonary, wound, gastrointestinal tract, postoperative, or urinary tract. We also have to consider the issue of timing, which was recently commented on in a review paper [83]. Patients who seek medical care and develop severe sepsis may not present at the ICU when the initial SIRS is at its most severe, but rather later when a predominant compensatory anti-inflammatory response syndrome (CARS) is evident. Strategies developed to fight organ failure in severe SIRS (for example, the animal model of lipopolysaccharide intravenous infusion) may be counterproductive in a human patient in the intensive care unit having CARS rather than SIRS.

When an injury induces ARDS we know exactly when the physiological insult occurred, which solves the issue of timing. Physical trauma is difficult to grade and the injury severity score (ISS) is often used clinically and for trauma studies [84]. For a physical trauma to induce ARDS or MOF it has to be substantial, and may be hard to control in an animal model. Human trauma of that severity tends to have many other relevant aspects that are difficult to account for such as traumatic brain injury or aspiration pneumonia. There is also the importance of pre-existing medical conditions, which is the same for all models of human ARDS but more pronounced when studying sepsis.

Burns are easier to grade as TBSA% is a direct measure of the extension of injury, but there may be relevant differences between a partial thickness and a full thickness burn. The presence of an inhalation injury may obscure the ARDS that results from the inflammatory host response induced by the burn of the skin, even though the relevance of the inhalation injury has been questioned in a study from our group [21].

The so called “two-hit” models are sometimes used, and are thought to resemble what happens when a vulnerable patient is infected after an injury. These models may resemble late-onset MOF, typically the patients who are immunocompromised after trauma and have organ failure secondary to sepsis. Because the second hit is an infective one, this model also carries all the negative aspects from the sepsis models in its later phases.
Perhaps the most striking feature of ARDS and MOF is how similar the syndromes are, regardless of the initiating event that may be as diverse as sepsis, trauma, pancreatitis, hypotension, or a transfusion reaction. This leads to a suspicion that there are common features and a common mediator of the syndrome that are the same, regardless of the initiating event.

This common feature may well be the overactivation of the innate immune system and systemic activation of granulocytes, a hypothesis supported by review papers.

Recently an interesting study was published [85] that looked at the reaction pattern of genes in leucocytes after mechanical trauma, burns, and infusion of LPS in healthy adults. They found that 80% of the granulocyte genome is up-regulated or down-regulated, which was unexpectedly high and called “a genomic storm”. Defined as the number of genes regulated in the same direction, this genomic storm was similar (correlation r=0.95) among the patients with mechanical trauma and those with burns, which suggested that there were identical reaction pathways in the leucocytes after those two events.

The authors also questioned the presence of a second hit, as they saw no genomic activation evidence of it. Probably most interesting at all, the similarities between infusion of LPS in healthy adults and trauma or burns were large (r=0.64), which suggested that the behaviour of leucocytes during an infusion of LPS resembles that after trauma or burns - a common pathway that may result in damage to organs.
1.9 Theories of multiple organ dysfunction syndrome (MODS), MOF, and the development of ARDS
The theory that activated leucocytes cause ARDS after trauma is not unique, and is complemented by an array of other theories reviewed by Pape et al. [15, 37].

Table 5. Theories about the aetiology of MODS

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Mechanism of the underlying theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage theory</td>
<td>Increased production of cytokines and other inflammatory mediators by activated macrophages</td>
</tr>
<tr>
<td>Microcirculatory theory</td>
<td>Prolonged hypovolaemic shock promotes MODS through inadequate global oxygen delivery, ischaemia reperfusion phenomena</td>
</tr>
<tr>
<td>Endothelial cell</td>
<td>Leucocyte interactions leading to remote organ injury</td>
</tr>
<tr>
<td>Gut hypothesis</td>
<td>Bacteria of gut origin or their products contribute to MODS</td>
</tr>
<tr>
<td>Anergy theory</td>
<td>Immune paralysis develops after overexaggerated initial inflammation and induces the MODS</td>
</tr>
<tr>
<td>“Two-hit” theory</td>
<td>Secondary injures to the inflammatory system in the “two-hit” model by factors such as surgical procedures and sepsis</td>
</tr>
</tbody>
</table>


The theory proposed in this thesis, that activated granulocytes causes ARDS (MOF), has no explicit name, but corresponds to the endothelial cell theory in the nomenclature used by Pape et al. I should prefer to name it “granulocyte-mediated theory” instead, to honour granulocytes as the key players. Even if the function of endothelial cells is of great importance in the process, the circulating leucocytes probably mediate the reaction. Stressed endothelial cells may also release factors of potential importance. In any case, it is probably best to look at this proposed theory as collateral damage to organs after the complex interaction of leucocytes and endothelial cells.

The macrophage theory proposes that the increased (rather altered) concentrations of cytokines in MODS, MOF, and ARDS come from the activation of macrophages. We know that macrophages and monocytes are capable of releasing these factors and that concentrations are increased or altered in organ failure [15]. These facts have led to the logical theory that the cytokines are the causative agents that mediate the syndrome. We also know that activated granulocytes activate monocytes and macrophages, so the macrophage theory is partly compatible with the leucocyte-mediated theory.
The microcirculatory theory proposes that an overall low flow state followed by overall reperfusion initiates what is described as an overall ischaemia-reperfusion injury at the microvascular level. This theory may certainly be true for some cases but not all, as circulatory failure is common but not obligatory when MODS, MOF or ARDS develop. For example, transfusion (the so called transfusion-related acute lung injury, TRALI) may induce ARDS without hypoperfusion. Of interest is that the regional ischaemia-reperfusion injury has been shown to be mediated, at least partially, by activated granulocytes that adhere and extravasate in the former ischaemic region at the time of reperfusion [86]. Even more interesting perhaps is that the overall increase in vascular permeability after hypovolaemic shock and reperfusion in rhesus monkeys was inhibited by blocking of antibodies to CD11b, which is the receptor used by granulocytes for activation at the time of endothelial adhesion [87]. These results confirm that the microcirculatory theory may be true for some cases of ARDS and is mediated by granulocytes that are activated by the ischaemia and reperfusion. A recent paper about another animal model pointed out that the organ injury-sparing effect of the so-called “postconditioning” procedure at reperfusion is explained by alterations in the functions of granulocytes that are related to the postconditioning [88].

The gut theory states that bacteria in the gut produce substances that are capable of activating the immune system, and these substances, or the bacteria themselves, are translocated from the gut mucosa and enter the circulation, probably more through the lymphatic system than the portal system [89]. The initiating event may be a state of low circulation or hypoxia in the gut, or a combination of the two. The support for the theory comes from an impressive series of animal studies that have shown that lymph from the gut after an ARDS-initiating event has the ability to, among other things, activate granulocytes in vitro [89], and that if the lymph is redirected (never entering the systemic circulation) this improves survival and abrogates lung injury [90-91]. The contrary, the two theories complement each other in that the gut theory may teach us about how granulocytes are activated and was recently reviewed by Deitch et al [92].

The anergy theory states that the initially strong immunological activation is followed by suppressed function of the immune system. This in turn leads to MODS, MOF or ARDS. Because suppression of the function of the immune
system is thought to develop somewhat later than the initial strong activation, it may be assumed that this theory deals predominantly with MOF of later onset, and that the stressor is a septic one. The immunosuppression may result in deadly infections from otherwise harmless microbes, such as staphylococci in normal flora growing on a central venous catheter. Babcock et al. reported the behaviour of leucocytes under such circumstances after burns, and showed that a decreased expression of CD11b and CD16 on granulocytes was associated with the development of sepsis later in the course of the illness [93]. We also know from many other studies that the granulocytic oxidative burst, chemotaxis, and phagocytosis are altered after burns and trauma [94]. This indicates that granulocytes may also have a role in sepsis-associated ARDS of late onset after burns, but the mechanism is the opposite. In the case of late onset it may be an inability of the granulocytes to clear bacteria that causes sepsis, and that in turn causes ARDS, as opposed to early ARDS where it seems that overactivated granulocytes induce aseptic collateral damage. The “two-hit” theory is essentially similar to the anergy theory, and postulates that the injured patient is in a vulnerable state. In this vulnerable state, a second hit (such as an operation or infection) that would normally be handled well may induce organ failure, often secondary to sepsis, but possibly also an aseptic reaction to further tissue damage such as operation, which would differentiate this theory from the anergy theory.

1.10 Summary of current knowledge

- Granulocytes are activated by burns and trauma.
- Activated granulocytes increase vascular permeability when they adhere to the endothelium.
- This increase in vascular permeability is mediated, at least in part, by HBP.
- ARDS is accompanied by increased vascular permeability and granulocyte transmigration from blood to the alveolus, and often occurs after burns and trauma.
- Important granulocyte functions such as the oxidative burst, chemotaxis, and phagocytosis are impaired in the later phase after burns or trauma.
- Histamine is suggested to be an important mediator of the increased vascular permeability after burns.
This leads us to the aims of this thesis.

1.11 Aims

The main aim of this thesis was to study granulocyte function after burns and trauma to find out the role played by granulocytes in processes such as development of increased vascular permeability and ARDS after injury.

The specific aims of the different investigations were (numbers unrelated to studies):

1. To find out if the expression of specific receptors is altered on leucocytes after a burn.
2. To find out if plasma concentrations of HBP, secreted from activated granulocytes after a burn, correlate with increased pulmonary vascular permeability and the decreased PaO\(_2\) : FiO\(_2\) ratio seen after burns.
3. To describe in detail the profile of dynamics of WBC after burns and relate the concentrations and changes to measured pulmonary vascular permeability and decreased PaO\(_2\) : FiO\(_2\) ratio after burns.
4. To see if immature forms of granulocytes are present in the circulation after burns.
5. To evaluate the possible value of concentrations of HBP sampled early after trauma, to predict ARDS.
6. To find out if the urinary concentration of histamine after a burn is compatible with its suggested role as a mediator of systemic increased vascular permeability after burns.
2 Patients, material, and methods

2.1 Ethics

Papers I and II.
The patients or their next of kin gave informed consent before sampling in accordance with a decision from the regional ethics review board (Linköping, Sweden).

Paper III.
The study was approved by the regional ethics review board (Linköping, Sweden). Patients or their next of kin gave informed consent before they entered the study.

Paper IV.
The study was approved by the regional ethics review board (Stockholm, Sweden). Patients or their next of kin gave informed consent before they entered the study.

Paper V.
Before the start of the study the regional ethics review board (Linköping, Sweden) was consulted and agreed that the study could be conducted without informed consent as only urine samples were being studied and patients were not identifiable.

2.2 Patients, study centres, and treatments

Papers I and II.
The study was conducted in the Burn Unit at Linköping University Hospital, a tertiary burn centre that serves a population of 3-4 million inhabitants for the treatment of patients who require specialised care of burns from the southern part of Sweden. Samples were collected during 1998 and 1999. The cytometer analysis in paper I was made in connection with the sampling and plasma was frozen and kept. Analysis of concentrations of HBP for paper II was made in
2008 and the manuscript prepared in 2009. Ten consecutive patients were included, and the inclusion criteria were a TBSA% greater than 15 % and age 18 years or older. Ventilatory assistance, nutritional support, sedation, and pain control were given in accordance with guidelines for modern treatment of burns at the time of the study and included fluid resuscitation in accordance with the Parkland formula, early excision and skin grafting, early enteral nutrition, and no prophylactic antibiotics.

Paper III.
The study was conducted in the Burn Unit at Linköping University Hospital, a tertiary burn centre. In 2010 it was decided by the Swedish National Board of Health that this centre would be one of two designated burn centres in Sweden, which increased the number of patients admitted. Samples were collected during 2010 and 2011. Twenty-two consecutive patients were included and the inclusion criteria were a TBSA% greater than 20 % and aged 18 years or older. Two patients died after an early decision not to continue with further treatment as the prognosis was not good. These two patients were excluded from further analysis. Ventilatory assistance, nutritional support, sedation, and pain control were given in accordance with modern guidelines for the treatment of burns [95] and included fluid resuscitation in accordance with the Parkland formula, excision and skin grafting in multiple sessions starting early, early enteral nutrition, and no prophylactic antibiotics.

Paper IV.
The study was conducted in the Central ICU at the Karolinska Hospital, Solna, Stockholm. Trauma patients aged 18 years or older with an expected stay in ICU of more than 24 hours, and when informed consent could be obtained from the patient or the next of kin, were eligible for inclusion. Data were collected between February 2007 and November 2008. Forty-seven patients were included and inclusion was not consecutive but depended to some degree on resources available, such as a research nurse. Only patients from whom we could obtain a plasma sample within 36 hours of their injury were included.

Paper V.
The study was conducted in the Burn Unit at Linköping University Hospital. Eight consecutive patients were included and the inclusion criterion was a
TBSA% greater than 10%. Ventilatory assistance, nutritional support, sedation, and pain control were given in accordance with guidelines for the treatment of burns at the time of the study (1998) and included fluid resuscitation in accordance with the Parkland formula, early excision and skin grafting, early enteral nutrition, and no prophylactic antibiotics. The samples were collected and analysed in 1998 and the manuscript was prepared in 2011.

2.3 Methods in Study I

Blood samples were drawn at the time of inclusion into the study and every consecutive day at 0600 in the morning until day 7 after the burn. Blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes and, after 100 µl had been transferred for flow cytometric analysis, the rest was centrifuged to plasma and frozen in minus 80° C.

Statistical analysis

We tested the time variations of the four different receptor expressions using Friedman’s analysis of variance (ANOVA) and Kendall’s coefficient of concordance. The receptors that showed significant changes (granulocyte CD11b, monocyte CD14, and granulocyte CD16) were analysed further. Three different multiple regression models for longitudinal data was used to analyse the significance of associations between the dependent variables: granulocyte CD11b, monocyte CD14 and granulocyte CD16 with the independent variables TBSA%, FTB %, patient number and day after injury for each of the receptor expressions.

2.4 Methods in Study II

The frozen plasma from study I was analysed with an enzyme-linked immunosorbent assay (ELISA) described elsewhere [35]. Briefly, plates were coated with a mouse monoclonal antibody directed against HBP. Plates were washed with phosphate-buffered saline plus 0.05 % Tween (a buffer) and blocked with 2% bovine serum albumin in phosphate-buffered saline plus 0.05 % Tween. Samples of plasma were then diluted and added to plates in duplicate and incubated for 30 minutes at 37° C. Calibrated samples of human HBP (0-600 ng/ml) were added in parallel to the plasma samples. After they had been washed, plates were incubated with a polyclonal rabbit antiserum against human HBP and bound antibodies were detected by incubation with peroxidase-
conjugated antibody against rabbit immunoglobulin G. Plates were developed and the optical density was determined at 420 nm.

**Statistical analysis**

The significance of change in concentrations of HBP over time was assessed by Friedman’s ANOVA.

### 2.5 Methods in Study III

Patients were included only if they arrived at the hospital within 24 hours after the burn, and sampling was started on arrival to ensure an early sample. Thereafter samples were taken at 8, 16, and 24 hours after the burn. For more convenient sampling times, sampling was then done at clock times 0600, 1400, and 2200 on days 2 and 3 after the burn. The time that elapsed from inclusion (if it was within 8 hours of the burn), therefore, and taking the second sample, may vary and the time that elapsed from the fourth sample (the one taken 24 hours after the burn) and the fifth sample (at 0600 in the morning on day 2 after the burn) may vary. After day 3 sampling was done at 0600 in the morning and on days 4, 5, 6, 9, 12, 15, 18, and 21.

Samples were collected in EDTA tubes and immediately spun to plasma and frozen in minus 80° C. Later the samples were analysed for the concentration of HBP in plasma as described in study II above. At the same sampling times we sent blood to the University Hospital laboratory for analysis of full blood count and differential count to assess the particle counts of granulocytes and mononuclear cells. Granulocytes were further subanalysed as immature (promyelocyte, metamyelocytic, or band forms), neutrophilic, eosinophilic, and basophilic.

Alterations in WBC and granulocyte counts were assessed as the maximal concentration minus the minimal concentration within 24 hours after the burn (\(WBC_{\Delta24}\) and \(granulocyte_{\Delta24}\)). As concentrations of HBP are highly variable and it seems that the peak may develop at different times in individual patients, we used the highest HBP concentration sampled within 24 hours of the burn for analysis (\(HBP_{\max24}\)). At the same sampling times we also measured the pulmonary vascular permeability index (PVPI) using the PiCCO system described below and further in the discussion. Chest radiographs were not taken at fixed times but as needed for clinical assessment.

**Statistical analysis**
Linear regression was used to assess the significance of possible associations between two continuous variables. We tested for the significance of differences between groups with the Mann-Whitney U test, and the significance of differences in proportions was assessed with Fisher's exact test. Multiple linear regression was used to assess the significance of associations between the dependent variable $PVPI_{\text{max24}}$ and the independent variables $WBC_{\Delta24}$, $HBP_{\text{max24}}$, age, and TBSA%.

2.6 Measurement of extravascular lung water and lung vessel permeability
The transpulmonary thermodilution method (PiCCO, Pulsion Medical Systems, Munich, Germany) is often used in intensive care for assessment of patients’ circulatory state [96].

The physical principle used is the Stewart Hamilton principle, which states that if a given amount of an indicator is infused, mixed in a volume, and the downstream concentration curve over time can be measured, this allows us to compute the flow via a certain formula. Instead of an indicator, the PiCCO uses a fixed amount of a cold fluid and measures temperature change. Cold sodium chloride is infused in a central venous catheter, close to or into the right atrium. The change in temperature measured in the femoral artery defines the flow that dissolves the indicator. This is used to calculate the cardiac output in the same way as in the gold standard, the pulmonary artery catheter. The difference is that the PiCCO system dissolves the indicator just before the right atrium and detects after the left ventricle – that is, transpulmonary thermodilution, and the pulmonary artery system dissolves just before or in the right atrium and detects in the pulmonary artery.
Fig. 5. Diagram of the circulation volumes in the thorax. With kind permission from Pulsion Medical Systems.

RAEDV = Right atrial end diastolic volume
RVEDV = Right ventricular end diastolic volume
PBV = Pulmonary blood volume
EVLW = Extravascular lung volume
LAEDV = Left atrial end diastolic volume
LVEDV = Left ventricular end diastolic volume

GEDV = Global end diastolic volume
GEDV = RAEDV + RVEDV + LAEDV + LVEDV
ITBV = Intrathoracic blood volume (GEDV + PBV)
PVPI = Pulmonary vascular permeability index
ITTV = Intrathoracic thermal volume

Intrathoracic thermal volume (ITTV) is the total sum of all the volumes shown in Fig. 5. The key to understanding how the PiCCO calculates the derived volumes is that it starts with the knowledge of the flow (from the Stewart Hamilton principle). With the knowledge of the mean transit time, the volume in which the indicator was dissolved can be derived, as flow = volume/time; cardiac output \cdot \text{mean transit time} \text{ is therefore the ITTV.}

When the series of volumes (mixing-chambers) comprising the ITTV is rewarmed by filling with blood at body temperature, the velocity of normalisation of temperature in the femoral artery is proportional to the volume of the largest of the different serial volumes that the indicator (temperature change) was allowed to mix in, and to the flow. The largest volume in the series of vessels and compartments in this case is the pulmonary thermal volume (PTV). So, the PTV is then derived from the normalisation velocity of temperature measured as the exponential decay time of the dilution curve and
the cardiac output (cardiac output \times \text{the exponential decay time}). From Fig. 5 it is easy to understand that \( \text{ITTV-PTV} = \text{GEDV} \). The results of empirical studies have shown that pulmonary blood volume (PBV) is 1.25 \times \text{GEDV} [97]. From all these measurements, only simple addition and subtraction are needed to compute intrathoracic blood volume (ITBV) and extravascular lung water (EVLW).

It is also essential for our understanding to note that because temperature is distributed immediately in the fine capillary bed of the lung, and also reaches the extravascular water, \( \text{PTV} = \text{PBV} + \text{EVLW} \). Knowing this it is logical that pleural effusions are not measured in thoracic volumes (as temperature is not dissolved in that space). An embolus in the pulmonary vasculature should also affect the measurement because it prevents the indicator from mixing in the whole thorax.

### 2.7 Methods in Study IV

Blood samples were collected in EDTA tubes and spun to plasma at 4°C at 1500 g for 10 minutes. Plasma was then transferred to another tube, once again spun at 3000 g for 10 minutes, and stored at minus 80°C before analysis. The ISS [84], acute physiology and chronic health evaluation (APACHE II) [98], and the sequential organ failure assessment score (SOFA) [99] were recorded on admission. ARDS was defined according to the American-European consensus criteria [24].

**Statistical analysis**

For comparison of the significance of differences between groups in continuous variables we used the Mann Whitney \( U \) test. Data are presented as median (interquartile range). The significance of differences between categorical variables was evaluated using Fisher’s exact test, and the significance of associations between plasma HBP and ISS, APACHE II, and SOFA were assessed with Spearman’s rank correlation. The predictive value of plasma HBP was assessed by calculation of the area under the receiver operating characteristics curve (AUC-ROC) where we compared patients with ARDS and those who did not have ARDS, and patients with severe sepsis and those who did not have severe sepsis, respectively. Probabilities of less than 0.05 were accepted as significant.
2.8 Methods in Study V

Urine was collected on admission when a catheter was inserted into the bladder (preadmission urine) and then every 6 hours for 48 hours after arrival in the Burn Unit. Urine samples were frozen and later analysed for the concentrations of histamine and the histamine metabolite methylhistamine using high performance liquid chromatography (HPLC), as described earlier [100-101]. Urinary creatinine concentration was measured directly at the University Hospital laboratory.

Statistical analysis

Data are presented as mean (95% confidence interval, CI). The significance of differences between controls and the first urinary fraction were assessed with the Mann Whitney U test. The significance of changes in histamine and methylhistamine concentrations over time was assessed using Friedman’s ANOVA.

2.9 Statistical software

Data were analysed with the help of STATISTICA (StatSoft® Inc, Tulsa, OK, USA). Version 9.0 was used in study I and version 7.0 in study II. Version 10 was used in studies III-V. Probabilities of less than 0.05 were accepted as significant.

2.10 Summary of the studies in the thesis

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Type of injury</th>
<th>No.</th>
<th>Severity (mean)</th>
<th>Type of study</th>
<th>Main outcome variable</th>
<th>Year of study</th>
<th>Mortality at 30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Burn</td>
<td>10</td>
<td>TBSA% 35</td>
<td>Observational</td>
<td>Leukocyte receptor expression</td>
<td>1998-1999</td>
<td>10%</td>
</tr>
<tr>
<td>II</td>
<td>Burn</td>
<td>10</td>
<td>TBSA% 35</td>
<td>Observational</td>
<td>Plasma HBP</td>
<td>1998-1999</td>
<td>10%</td>
</tr>
<tr>
<td>III</td>
<td>Burn</td>
<td>22</td>
<td>TBSA% 40</td>
<td>Observational</td>
<td>Plasma HBP, WBC dynamics and PVPI</td>
<td>2010-2011</td>
<td>18% (27% ICU mortality)</td>
</tr>
<tr>
<td>IV</td>
<td>Trauma</td>
<td>47</td>
<td>ISS 26</td>
<td>Observational</td>
<td>Plasma HBP and ARDS</td>
<td>2007-2008</td>
<td>13%</td>
</tr>
<tr>
<td>V</td>
<td>Burn</td>
<td>8</td>
<td>TBSA% 24</td>
<td>Observational</td>
<td>Urine histamine and Methylhistamine</td>
<td>1998-1999</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Study I and II are based on the same series of patients. TBSA% = percent total body surface area burned, HBP = heparin binding protein, WBC = white blood cell count, PVPI = pulmonary vascular permeability index, ICU = intensive care unit, ARDS = acute respiratory distress syndrome.
3 Results

3.1 Study I

The expression of granulocyte CD11b is shown in Fig. 6. There was an early increase followed by a decrease to the high points of the reference range by the end of the first week (the end of the study). This dynamic change was significantly different from a variation that could have occurred by chance (p<0.05).

Fig. 6. The expression of granulocyte CD11b after burn injury. MFI, Mean Fluorescence Intensity. Squares indicate the mean, the box SD and the whiskers 95% CI. From study I.

The expression of granulocyte CD16 is shown in Fig. 7 and there was a sharp decrease that was sustained during the study (Fig. 7), p<0.05.
Fig. 7. Variations over time of granulocyte CD16. MFI, Mean Fluorescence Intensity. Squares indicate the mean, the box SD and the whiskers 95 % CI. From study I.

The changes in monocyte CD14 are shown in Fig. 8. There was an increase followed by a slight decrease (p<0.05). By the end of the study the expression was still increased compared with that of healthy controls.
Fig. 8. Variations over time of monocyte CD14. MFI, Mean Fluorescence Intensity. Squares indicate the mean, the box SD and the whiskers 95% CI. From study I.

The different receptors were included in multiple linear regression analyses to explore which of the receptors (as the dependent variables) were associated with the independent variables of size of injury (TBSA% and FTB%). Because of the suspected dependency of receptor expression in relation to time, the day after the trauma was included as an independent variable. Because of the suspected interpatient variability of the reaction, the identity of patients was included as a nominal variable. The results are summarised in table 7.

*Table 7. Multiple regression coefficients that explored different determinants of the respective leucocyte receptor expression*

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Patient No.</th>
<th>p value</th>
<th>Day after injury</th>
<th>p value</th>
<th>TBSA%</th>
<th>p value</th>
<th>FTB%</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocyte CD11b</td>
<td>-16.5</td>
<td>0.05</td>
<td>-18.0</td>
<td>0.08</td>
<td>0.50</td>
<td>0.07</td>
<td>-0.58</td>
<td>0.04</td>
</tr>
<tr>
<td>Granulocyte CD16</td>
<td>-0.25</td>
<td>0.05</td>
<td>-0.13</td>
<td>0.22</td>
<td>0.56</td>
<td>0.03</td>
<td>-0.81</td>
<td>0.002</td>
</tr>
<tr>
<td>Monocyte CD14</td>
<td>-0.30</td>
<td>0.02</td>
<td>-0.30</td>
<td>0.006</td>
<td>0.13</td>
<td>0.61</td>
<td>-0.31</td>
<td>0.23</td>
</tr>
</tbody>
</table>

The coefficients that are highlighted indicate that there is a significant association with the respective receptor. TBSA% = percent total body surface area burned, FTB% = percent full thickness burn.
3.2 Study II

There was a significant change over time of plasma HBP after a moderate to severe burn (p<0.05) (Fig. 9).

![Box and whisker plot showing mean, SE, and 95% CI. From study II.](image)

3.3 Study III

There was no difference over time in plasma HBP (Fig. 10).

![Box and whisker plot showing mean, SE, and 95% CI. From study III.](image)
There were large variations in numbers of WBC and granulocytes in blood over time (Figs. 11 and 12).

Fig. 11. White blood cell count (WBC) over time after injury, note the change of time scale on the X-axis after 72 hours. From study III.

Fig. 12. Polymorphonuclear neutrophil (granulocyte) concentration in plasma over time after injury, note the change of time scale on the X-axis after 72 hours. From study III.

There were significant correlations between the variables $\text{PVPI}_{\text{max}24}$ and $\text{WBC}_{\Delta24}$ and granulocytes$_{\Delta24}$, but no correlation between $\text{HBP}_{\text{max}24}$ and $\text{PVPI}_{\text{max}24}$ (Fig. 13).
Fig. 13. Scatterplots showing associations between PVPI$_{\text{max}24}$ and the variables, WBC$_{\Delta24}$ (A), granulocyte$_{\Delta24}$ (B), and HBP$_{\text{max}24}$ (C). WBC$_{\Delta24}$; $r=0.77$ $p<0.001$ granulocyte$_{\Delta24}$; $r=0.76$ $p=0.001$ HBP$_{\text{max}24}$; $r=0.28$ $p=0.324$. From study III.
Fig. 14. Immature forms of PMN during the study. Immature forms include metamyelocytes, myelocytes and bandforms. Open circles are outliers. Data presented as median (interquartile range). From study III.

### 3.4 Study IV

There were differences in early HBP, as well as in ISS, between patients who did, and did not, have ARDS during their stay in ICU (Fig. 15)

![Graph showing differences in HBP and ISS between No ARDS and ARDS groups.](image)

Fig. 15. There were differences in early HBP as well as ISS between the groups developing and not developing ARDS. ARDS, acute respiratory distress syndrome. HBP, heparin binding protein. ISS, injury severity score. From study IV.

The predictive value of HBP, here depicted as a ROC curve (study IV), is seen in Fig. 16A. It is complemented by Fig. 16B, which is the same analysis but with ISS as the proposed predictor of ARDS.
Table 8 shows the explanatory values of the variables early HBP and ISS combined with age and sex, when used in a logistic regression analysis in which ARDS was compared with no ARDS as the dependent variable. From the combined model of the prediction of ARDS that came from this logistic regression it was possible to construct a further ROC curve with the combined predictive power of HBP and ISS, which is shown in Fig. 17.

Table 8. Multiple logistic regression that explored possible determinants of ARDS

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient of regression</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISS</td>
<td>4.78</td>
<td>0.03</td>
</tr>
<tr>
<td>HBP</td>
<td>3.28</td>
<td>0.07</td>
</tr>
<tr>
<td>Sex</td>
<td>0.18</td>
<td>0.67</td>
</tr>
<tr>
<td>Age</td>
<td>0.09</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Fig. 16. ROC-curves for early HBP (A) and ISS (B) when used for prediction of ARDS-development after trauma. The respective areas under curves are 0.748 (HBP) and 0.679 (ISS). Calculated from data in study IV.

Fig. 17. The ROC-curve when early HBP and ISS are used together in a combined model to predict ARDS after trauma. Area under the curve is 0.81. Calculated from data in study IV.
3.5 Study V

Urinary histamine and methylhistamine over time in relation to the healthy controls is shown in Fig. 18. There was a difference in urinary histamine between the control and the patients' first sample ($p=0.002$). There was no difference for methylhistamine ($p=0.98$). There was no significant variation over time in any of the variables assessed by Friedman’s ANOVA.

Histamine and methylhistamine concentrations in urine corrected for creatinine excretion are shown in Fig. 19. There were no significant variations over time.

---

**Fig. 18.** Urine histamine and Methylhistamine after burn injury. Mean and CI. From study V.

**Fig. 19.** Urine Histamine and Methylhistamine corrected for creatinine-excretion after burn injury. Mean and CI. From study V.
4 Discussion

4.1 The state of granulocytes in the circulation and possible extravasation after injury

In study I we initially found increased expression of receptor CD11b on granulocytes after a moderate to severe burn. This is in line with previous investigations [102-104]. We know that CD11b is a receptor critically involved in the activation of granulocytes when they are firmly adherent to the endothelial cells. The total process of transmigration of granulocytes is complex and involves several steps. The increased expression of CD11b may reflect that granulocytes are more likely to adhere, but rolling is a prerequisite for firm adhesion and the principal receptor for adhesion is CD11a so the fact that CD11b is increased does not imply a state of massive extravasation of granulocytes. The binding of CD11b to the endothelial cells probably has more of an activating function on the granulocyte, in contrast to CD11a that mediates the mechanical binding.

The actual extravasation of leucocytes is very hard to document in humans, but in study III we suggest that it may be estimated indirectly by recording the decrease in the number of leucocytes that are still circulating. In study I no differential counts were measured, and WBC were measured only once daily and this did not start as early as in study III. With this low resolution in time and the late arrival of the first samples it was not possible to document exactly the dynamics of leucocyte counts after burns. Despite this we could see a rise and fall of WBC (not shown), which corroborated the results of earlier studies [21]. In study III we confirmed a distinct rise followed by a fall to low normal, or subnormal, concentrations within 24-48 hours. In study III the process was documented in more exact detail by better resolution in time and with a differential count.

We believe that the difference between the maximal and the minimal WBC/granulocyte count may correlate to the number of extravasating granulocytes. A small part of this decrease may also be accounted for by the fluid kinetics after burns. For example, the Hemoglobin concentration is often in the range of high normal, 150-160 g/l, on arrival and decreases to 100-120 g/l after early resuscitation (seen in study III but data not shown). This dilution effect may account for a small part of the early decrease in the WBC. We can present no proof that the decrease in the WBC count corresponds to the amount extravasated. All our evidence is circumstantial. We know that before the burn
WBC are most likely to be within the normal range (4-9 x 10^9/l). Only a few hours after the burn the concentration is often double or even more. The median time to first sample in study III was 8 hours after the burn, and median WBC count after 8 hours was 18.1 x 10^9/l. There was a tendency for WBC counts to be lower with increased time to the first sample (not shown), indicating that the maximal WBC count was missed by many patients. The WBC profile from study III is shown in Fig. 11. The highest recorded WBC count in the study was 36.6, and was taken 2 hours after the burn.

From study I it was also evident that granulocyte CD62L did not show a reproducible expression pattern after burns. This receptor is constitutively expressed on circulating granulocytes and earlier investigations have confirmed so called shedding, where the receptor is cleaved and enters the circulation in soluble form, upon activation of the granulocyte in surgical patients with SIRS [105]. It is important to remember, when trying to draw conclusions from expressions of this and other receptor expressions, that the life of a granulocyte is rather short in the circulation even in the normal healthy subject. The time spent in the circulation in a state of SIRS after a burn may be even shorter, somewhat supported by the rapid decline of leukocytes in study III. It is also likely that granulocyte expressing CD62L are more prone to so called secondary capture, thereby leaving the CD62L-negative cells in the circulation for sampling. Secondary capture is the process where the granulocyte attach to an already adherent granulocyte, a process that may be of physiological importance [106] and promote removal of granulocytes expressing CD62L.

This translates to a situation where a postulated altered expression of CD62L on granulocyte is not for sure possible to detect by the methods used in study I. Hence the results found are compatible with a situation of increased rolling, adhering and extravasation but no further definitive conclusions may be drawn from the absence of variation of granulocyte CD62L.

The other two receptors studied in study I, granulocyte CD16 and monocyte CD14, are not involved in the extravasation and will be discussed later.

4.2 Functional state of circulating leucocytes in relation to time after burns and immunosuppression

Immature forms of granulocytes appear in greater proportion than normal (reference value is 0) after burns and the concentrations increased even more after 7 days in study III (Fig. 14). This is not shown earlier in human burns. The reason may be that a large proportion of newly mature granulocytes are released
from the bone marrow after the burn, of which many are extravasated early and this explains the rapid rise and decline. Release of new mature granulocytes may be hampered by the fact that the normal process of maturation of granulocytes takes roughly two weeks, and also the possibility of suppression of bone marrow induced by the burn [107-108], which would explain the granulocytopenia. The secretory vesicles are lacking in immature forms because they are the last to form in the maturation process. They may even be formed by endocytosis after granulocytes have been released into the circulation [33]. This may be of clinical importance as fusion of secretory vesicles with the cellular membrane is part of the process in which certain adhesion molecules are upregulated when granulocytes are activated - for example, CD11b [33]. It was evident from study I that granulocyte expression of CD11b was initially increased, and by the end of the study (after seven days) the expression had declined and come close to control values (Fig. 6). From study III it was evident that the number of immature granulocytes started to increase from day 7 onwards. These two circumstances may be connected to some degree. The reduced ability of granulocytes to upregulate CD11b would be explained by the immaturity of the circulating granulocytes, a finding that parallels that of Rodeberg et al. [109] who found decreased CD11b concentrations in vesicles of granulocytes later after burns. This may partially explain the immunosuppression after burns, which is supported by the fact that it has been shown that the onset of sepsis in the late phase after burns was preceded by a decrease in CD11b expression by granulocytes [93].

Our finding in study I that granulocytes and CD16 expression showed a rapid and prolonged decline is thought to explain why the granulocyte capacity for Ig-mediated phagocytosis (mediated by CD16) declines after a burn [103]. Similarly, it is thought that the early increase and the later decrease of granulocyte CD11b, explains that complement-mediated phagocytosis is increased early after a burn (mediated by CD11b) [103-104].

Indirect evidence of the inability to increase the number of leucocytes after burns is the fact that these patients undergo massive surgical tissue trauma (revision and skin grafting) during the initial week after the burn. In a patient with normally-functioning bone marrow this would lead to massive leucocytosis, which we did not see in our series (Fig. 11, study III).
4.3 Difference between granulocyte CD11b expression and CD16 expression

Increases in cell-surface receptor expression are often caused by exocytosis of a granule that previously had the receptor expressed on its inside. After exocytosis, the receptor will be located on the outer surface of the cell. This is the case with granulocyte CD11b and CD16 [32], so it is interesting to look at the different subcellular localisation of CD11b and CD16 before the granulocyte is activated. There are four types of granules in granulocytes. We think that some of the circulating granulocytes may lack secretory vesicles. It was shown earlier that CD16 is contained exclusively in walls of secretory vesicles but not the other types of granules. CD11b is contained in the walls of secretory vesicles, tertiary granules, and secondary granules [33]. This is compatible with the fact that the burn induces massive early release of granulocytes, of which many extravasate early. This first wave of released and infiltrating cells was not captured by study I because sampling started too late. The rest of the supply of granulocytes in the bone marrow was stressed out in the circulation as partly immature granulocyte or bandforms, and these lacked secretory vesicles so had decreased CD16 expression. The increased expression of CD11b was still possible because it is possible to transfer it from the tertiary and secondary granules to the surface of the cells. Tertiary and secondary granules are formed earlier in the maturation process.

4.4 Determinants of leucocyte receptor expression

In study I we reproduced some results about the response of granulocytes after burns [102-104], and brought some new information on the topic, which related to the respective impact of the TBSA% as opposed to the impact of FTB% on the expression of the receptors being investigated. Even though the number of patients and the day after the burn showed trends (but not significant regression coefficients), the interpretation is still that it is likely that there is an individual factor leading to different degrees of expression in different patients with the same sort of burn. It is also likely that there is variation over time. Such interpretations are not controversial (although not all were significant in our small series).

More interesting is the idea that the reaction patterns seen in granulocyte CD11b and granulocyte CD16 show three significant results and one trend when related to the variables TBSA% and FTB%. This suggests that the burned tissue has an effect on the granulocyte that alters (increases and decreases) the respective
receptor expression. The association with full thickness burns was negative, however, which should correspond to a decrease in the expression of the receptor when the size of the full thickness burn was increased. This was unexpected and is discussed further below.

The reaction pattern of monocytes and the role of monocytes in organ failure after injury have not been studied much before. Monocytes react somewhat more slowly than granulocytes and enter a site of inflammation later. It is interesting to note, though, that the first effect of granulocytes is thought to “pave the way” for monocytes that are about to infiltrate the tissues [110]. The significant early increase in monocyte CD14 expression that we saw is interesting but, considering the lack of other studies, it is difficult to put into context. It is clear though that toll-like receptor 4 (TLR-4), to which CD14 is a co-receptor, plays an important role in Gram negative sepsis. TLR-4 is the receptor for the endotoxin lipopolysaccharide.

4.5 Possible differences between partial thickness and full thickness burns

To explore the contributions of TBSA% and FTB% to granulocyte receptor expression fully it is essential to appreciate that FTB% is a subset of TBSA%. This means that, even though FTB% and TBSA% are positively correlated with each other (study III, data not shown), an increased FTB% must, for a particular patient with a fixed TBSA%, mean a decreased partial thickness burn % (PTB%) and vice versa. This was also the case in study III (data not shown).

The simple mathematical relation between the three different measures of the size of the burn is TBSA% = PTB% + FTB%. The PTB% is not often used to measure size, which may be misleading as there are reasons to think that there may be relevant differences between the immunological and physiological impacts of a partial thickness, as opposed to a full thickness, burn on a patient. When we included both TBSA% and FTB% in the multiple regression analysis (study III) as variables for the size of the burn, with the aim of describing their respective impact on PVPI_{max24}, the result was somewhat unexpected. The contribution of FTB% was not significant but negative (an increased FTB% would lessen the PVPI_{max24}, the opposite to that of TBSA%) (Table 9). This is unexpected in physiological terms, but in fact the finding may fit with the results from study I where we found that the increase in surface receptor CD11b on granulocytes after burns was positively associated with TBSA% but negatively associated with FTB% (a trend for TBSA% and significant for FTB%). As
CD11b is the receptor used to activate granulocytes when they adhere to endothelial cells, it may follow that a larger FTB% will decrease PVPI as we think that it is the adhering, activated granulocytes that cause the increase in PVPI.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBP_{max24}</td>
<td>0.26</td>
<td>0.18</td>
</tr>
<tr>
<td>WBC_{Δ24}</td>
<td>0.93</td>
<td>0.003</td>
</tr>
<tr>
<td>Age (years)</td>
<td>-0.14</td>
<td>0.49</td>
</tr>
<tr>
<td>TBSA%</td>
<td>0.33</td>
<td>0.21</td>
</tr>
<tr>
<td>FTB%</td>
<td>-0.48</td>
<td>0.13</td>
</tr>
</tbody>
</table>

PVPI=pulmonary vascular permeability index, HBP=heparin binding protein, WBC=white blood cell count, TBSA%=percentage total body surface area burned, and FTB%=percentage full thickness burn.

Table 9. Contribution to PVPI_{max24} in a multiple linear regression analysis, recalculated from study III

By definition a partial thickness burn has some intact vessels, and therefore circulation, but a full-thickness burn has no circulation to the skin because the tissues are coagulated. This may explain why circulating leucocytes may pass through a partial thickness burn, have their CD11b upregulated, and so contribute to respiratory failure distant from the burn. Because the skin of a full-thickness burn is devoid of vessels, the same is not true and the FTB% may instead be inert. However, a patient with a large area of the total burn FTB% has less PTB% and may therefore be at less risk of increased PVPI.

To look further into this potential relation we introduced the variable PTB% as defined above. PTB% is simply TBSA% minus FTB%. If it is true that the partial thickness burn contributes the most to increased PVPI, and that the FTB is inert, then this may be further explored by introducing the new variable PTB% instead of TBSA% into the multiple linear regression. There was some support, and the (not significant) negative contribution of the FTB decreased from -0.48 to -0.12, when the PTB% was introduced instead of TBSA% (Table 10).
Table 10. Contribution to $\text{PVPI}_{\text{max24}}$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{HBP}_{\text{max24}}$</td>
<td>0.26</td>
<td>0.18</td>
</tr>
<tr>
<td>$\text{WBC}_{\Delta 24}$</td>
<td>0.93</td>
<td>0.003</td>
</tr>
<tr>
<td>Age</td>
<td>-0.13</td>
<td>0.49</td>
</tr>
<tr>
<td>PTB%</td>
<td>0.26</td>
<td>0.21</td>
</tr>
<tr>
<td>FTB%</td>
<td>-0.12</td>
<td>0.65</td>
</tr>
</tbody>
</table>

PVPI = pulmonary vascular permeability index, HBP = heparin binding protein, WBC = white blood cell count, PTB% = percentage partial thickness burn, and FTB% = percentage full thickness burn.

This particular coefficient of regression was not significant before the switch of variables, which lessens the value of the observation. The fact that it is not significant after the switch of variables would be expected if it is true that the FTB is inert with respect to CD11b upregulation, extravasation, and increased PVPI. The interpretation of this is that research into the physiology of burns with respect to activation of leucocytes, pulmonary vascular permeability, and ARDS may benefit from abandoning the old concepts of TBSA% and FTB% as the factors that define the size of the burn and instead use PTB% and FTB% to define it. This, of course, needs to be further validated, as the regression coefficients for PTB% and FTB% were relatively small and not significant.

Since we found the possibility of a stronger relation between granulocyte activation and the partial thickness burn, and discovered that the full thickness burn may be inert in this aspect, we returned to the data from study I and constructed the variable partial thickness burn ($\text{PTB} = \text{TBSA} - \text{FTB}$). We then exchanged TBSA% for PTB% and did the multiple regression analysis from study I again. It was interesting that the somewhat unexpected significant negative regression coefficients from study I were substantially decreased in effect size by this. The regression coefficient for FTB% was not significant after the switch of variables, which would also be expected if it is inert with regard to granulocyte activation.
Table 11. Regression coefficients from multiple regressions with PTB\% instead of TBSA\%, recalculated from the data in study I

<table>
<thead>
<tr>
<th>Receptor</th>
<th>PTB%</th>
<th>p</th>
<th>FTB%</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>granulocyte</td>
<td>0.21</td>
<td>0.07</td>
<td>-0.14</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Table 11 may be compared with table 7 above in the results section referring to study I. The interesting point is that the effect of the former significant negative contribution of FTB\% to granulocyte CD11b expression is now substantially decreased in size (decreased regression coefficient) and not significant. This is weak support for the same conclusion as in study III, that the FTB is inert in a granulocyte activation sense and therefore the PTB is a better definition of the size of a burn. This needs to be evaluated further. Some support for the conclusions may be found in the study by Shimitzu et al in rats [43]. They found that a FTB produced a large negative inhibition pressure that lasted less than 50 minutes, but the PTB produced no significant negative inhibition pressure. Despite this, the oedema was more pronounced in the PTB. Their conclusion was that an increased vascular permeability must have been responsible for the oedema in the PTB, and that the FTB may have been devoid of circulation (less oedema despite of the large negative inhibition pressure) as a result of coagulation of vessels, leading to what they call a reperfusion injury in the PTB but not in the FTB.

4.6 Plasma level of HBP after injury and its possible role for increased permeability and ARDS

In study II we found a distinct increase in the plasma concentration of HBP after a burn. We drew the conclusion that this was a consequence of massive early adhesion of granulocytes to the endothelial cells and spillover of HBP into the systemic circulation. Because HBP mediates an increase in vascular permeability when deposited on endothelial cells, we suggested that HBP may be the mediator of the increased vascular permeability after burns. In study III we tried to describe the dynamic change in the plasma concentration of HBP after burns more exactly, and also to correlate it with the measured vascular permeability, but we could not confirm the considerably increased concentrations from study II (Fig. 9). It is not entirely clear why the results are different. Possible explanations are as follows:
The samples used in study I were collected in 1998-1999 and stored at -80°C. It is unlikely that the samples were affected and concentrations of HBP altered by storage as HBP is a stable protein (Heiko Herwald, personal communication). However, it may be that the treatment of burns has changed. In 1998-99 it was common to revise the whole burn early in one extensive surgical session. Now it is more common to divide the revision into two or three sessions during the first 4-6 days. This lessens the degree of early surgical trauma, the use of blood products, and the effects of relative hypovolaemia in relation to anaesthesia and bleeding.

The median TBSA% was larger in study III (Table 6), which possibly explains the sustained increased concentration during the study (14-18 ng/ml), unlike study I in which concentrations declined to the range 8-12 ng/ml after the initial peak.

Blood products used in 1998-1999 (study I) were a mixture of leucocyte filtered and non-leucocyte filtered. In study III all blood products used were leucocyte filtered.

There were no dynamic changes in the plasma concentrations of HBP in study III, so one of the conclusions is that the systemic concentration of HBP does not mediate increased vascular permeability after a burn. It may still be that HBP, released from secretory vesicles in close proximity to the endothelium when granulocytes adhere, acts locally in a paracrine fashion and exerts this effect. For HBP to do this in vitro, a concentration in the range of 25-75 µg/ml [65] had to be used, which is much greater than the concentrations measured in plasma in our clinical studies and those of others (5-75 ng/ml). Concentrations measured in the systemic circulation may therefore not suffice to mediate the increase in vascular permeability that may still occur directly when there is close interaction between granulocytes and endothelial cells. It may even be that a small closed compartment is developed under the adherent granulocyte, and the concentration in such a compartment may be substantially higher than in free-flowing conditions.

The circumstances discussed previously concerning the immature state of circulating granulocytes in the later phase after burns may also explain the fact that we found stable and relatively low concentrations of HBP in plasma during the later phases of studies II and III. This follows from the fact that HBP is contained in secretory vesicles, which are lacking in immature forms of granulocytes, and so no release is possible during later phases of surgical stress.
If a previously healthy person was subject to the massive surgical stress caused by a skin revision after a burn, it would probably result in leucocytosis, granulocytosis, increased granulocyte CD11b and, possibly, an increase in the concentration of HBP in plasma. These reactions may be both adequate to protect from infections and impose an increased risk of organ failure, but patients with burns may have lost these reactions to some degree. The term CARS (Compensatory anti-inflammatory syndrome) is sometimes used for this state that may arise in the later phase after an injury and contrasts to the early SIRS.

4.7 Transpulmonary thermodilution measures of pulmonary vascular permeability

The reliability of the transpulmonary thermodilution system has been confirmed in a series of studies. The system for the measurement of cardiac output is often compared with the gold standard, the pulmonary artery catheter, and has been shown to be accurate [111-112]. As far as EVLW is concerned, the gold standard is gravimetric calculation of the amount of water in lungs, and PiCCO have been shown to work well both in animal models and a human necropsy examination [96, 113]. Because EVLW is confirmed by comparison with other reliable methods, and EVLW is derived from ITBV, the accuracy of ITBV may also be considered to be verified by the same studies. Because pulmonary oedema is a common problem in intensive care, the measure of EVLW is interesting. The volume itself has to be adjusted to the size of the patient and this is often indexed to (divided by) body weight, which yields the measure EVLWi. This is useful if you are interested in the actual amount of extravasated fluid in the lungs - for example, following the clinical condition of a specific patient. However, EVLWi does not automatically correspond to pulmonary vascular permeability changes as the amount of extravasated fluid also depends on the filling pressures (see the Starling equation above in 1.6.1). We may indicate the filling pressures with the measure ITBV. When we use EVLW and index it to (divide by) ITBV we get a measure of the amount of extravasated fluid adjusted for the filling pressure. This is a measure of the so-called pulmonary vascular permeability index (PVPI). It was originally defined as PVPI = EVLW/ITBV. Later publications modified it, and used PBV in the numerator but as ITBV = 5 x PBV the different measures differ only by a factor 5.
It was recently shown clinically that the PVPI was an excellent way to define whether patients had or did not have ARDS [114], and that this measure was not only a surrogate endpoint but correlated with hard endpoints [115]. Some authors have even proposed that PVPI should be used to define ARDS status. This could indeed be an elegant solution, as increased pulmonary vascular permeability is a hallmark of ARDS but not clinically applicable or useful as measurement of PVPI is expensive, time-consuming, and invasive. These facts in themselves carry a paradox. Usually when we try to define clinical status we use a simple test and relate it to a gold standard, which is more accurate but in some ways not applicable to all patients (for example, expensive, invasive, or time-consuming). Here we have the opposite: a clinically useful, simple definition or ARDS that is, if not gold standard, at least the most (the only) accurate definition. We have a more invasive, and probably more accurate, method and have to relate it to the standard. We suspect that the research on ARDS is hampered by these circumstances and would benefit from a usable and relevant gold standard for research purposes. The simpler clinical definition that is used may then be related to this gold standard. Similar issues have been discussed recently [116-118].

4.8 Alterations in leucocyte concentrations after burns and their relation to increased pulmonary vascular permeability

It is obvious from study III that the number of leucocytes, and the subgroup granulocytes, are subject to extreme and predictable alterations after a burn. As we suspect that the maximal number of granulocytes, minus the minimal number, in - for example - 24 hours, may correspond to the total systemic amount of extravasation, and we know from preclinical studies that adherent granulocytes increase vascular permeability [119], we investigated the possible relation between granulocyte$_{\Delta 24}$ and PVPI$_{\text{max24}}$. The relation that we found was strong. It was as strong when we used WBC$_{\Delta 24}$ instead of granulocyte$_{\Delta 24}$, which is important because WBC count is an inexpensive, easy, and readily available laboratory marker already in use all over the world. This makes our findings clinically easy applicable. The relation found does not prove a cause and effect relation. It may be a confounder - the burn injury - that causes both the increased PVPI and the WBC$_{\Delta 24}$. When we tried to adjust for this effect in a multiple linear regression analysis, including WBC$_{\Delta 24}$, TBSA%, and age, it was evident that the only
independent variable included in the regression that explained the increased PVPI was $\text{WBC}_{\Delta24}$. TBSA% did not make a significant contribution. This does not prove a cause and effect relation, but suggests that $\text{WBC}_{\Delta24}$ is a more important variable than TBSA%.

4.9 Relations among increased pulmonary vascular permeability, ARDS, duration of hospital stay, and mortality

It is obvious from study IV and other studies that patients with ARDS do worse in the ICU than patients who do not have ARDS. Their duration of stay is longer [study IV], their functional status after intensive care is worse (particularly if they have additional organ failure) [3, 120], and their mortality is higher [2]. Whether or not this is causal or an effect of a more severe trigger diagnosis is not easy to know for certain. Study IV did not show increased mortality among the patients with ARDS compared with patients who did not have ARDS. The same was true for study III, where no difference in mortality could be detected between the groups with a low P:F ratio as opposed to a normal P:F ratio. As they were small, exploratory studies they did not have the power to detect such differences. As far as duration of stay in ICU was concerned, there were differences in both studies, and patients with ARDS after trauma and patients with decreased P:F ratio after burns stayed longer in the ICU.

The functional status after rehabilitation is unknown in studies III and IV. Others have shown that patients in critical care with ARDS, high EVLWi and PVPI have increased mortality [115], and a systematic review of 670 patients came to the conclusion that EVLWi could be used as a predictor of mortality in a mixed ICU (including burns) [121]. The patients in study III did not by definition have a diagnosis of ARDS (as early chest radiographs were lacking in some cases, and in a few were present but showed no bilateral effusions). The possible connection of our indirect indicators for severity of disease (decreased P:F and high PVPI) and poor prognosis therefore remains to be investigated. The above studies were conducted in a general critical care unit and we must appreciate the possible differences between burns and critical care in general. In study IV the outcome measure was ARDS, and we think that it is reasonable to conclude that their prognosis was affected, although this was not detected (not surprisingly taking into account the low power of the study).
4.10 Plasma HBP as a prognostic marker of respiratory failure after severe trauma

In study IV we investigated the possible correlation between early concentrations of HBP in plasma in severely injured patients with ARDS as a consequence of the injury. We found that intensive care patients who developed ARDS did have increased concentrations of HBP in plasma soon after injury. Most patients who developed ARDS did not fulfill the criteria at the time that the plasma was taken, which made us think that there may be a causal relation between accumulation of pulmonary granulocytes (which was presumably paralleled by release of secretory vesicles and increase in concentrations of HBP in plasma) and the development of ARDS. As study IV was an observational study, it was not possible to establish a causal relation.

It has been shown previously, and in study IV itself, that more severely injured patients have a higher risk of developing ARDS, so the first question that arises is whether the predictive value of HBP concentrations is independent of the severity of the injury or not. As shown in Fig. 4 (in the paper itself, study IV) there was no strong association between ISS and early concentrations of HBP. This suggests that the information carried by one variable is not the same information as is carried by the other variable. We know from Figs. 15 and 16 that ISS and early concentrations of HBP perform fairly well in the prediction of ARDS one at a time in a univariate analysis. When we do a multiple logistic regression analysis with ARDS as the dependent variable and ISS, early concentration of HBP, age, and sex as independent variables it shows that the explanatory value of the model is increased compared with the univariate models (not shown), which strengthens the suspicion of independence. In other words each of the variables concentration of HBP, and ISS, carry some independent information about the risk of the development of ARDS.

It is also possible to retrieve ROC curves from the logistic regression and for the individual variables themselves. It is clear that the two variables themselves, ISS and early concentration of HBP, have individual discriminatory ability and that this ability is increased when we use the two tests in a combined model, reaching an AUC in the ROC analysis of 0.81 (Fig. 17). This additive effect also strengthens the suspicion that ISS and early concentration of HBP are fairly independent explanatory factors for the development of ARDS after severe trauma. In the combined model, the p-values of the two explanatory variables
ISS and early concentration of HBP are still fairly low but, in the case of HBP, slightly above 0.05 (table 8). It is also important to note that it is not expected that ISS and early concentration of HBP would be totally independent of each other. On the contrary, it is likely that a larger injury induces more pronounced activation of granulocytes and release of HBP. The ISS was originally designed to predict death after trauma, but was shown in study IV and others [122] to correlate with the development of ARDS as well. These facts indicate, therefore, that the concentration of HBP is independent of the ISS for the prediction of ARDS, but a causal relation with ARDS remains to be investigated.

4.11 Role of histamine in systemic vascular permeability after burns

Study V was conducted to confirm the postulated, but poorly documented, idea that histamine is the most important mediator of the systemic increase of vascular permeability after burns. The results showed a significant yet slight increase in the urinary concentration of histamine (Figs. 18 and 19). These are a mirror of the concentrations in plasma [123]. The results parallel similar studies in animals [43, 63]. The question is whether this increase is physiologically relevant. We think that the change would have been larger and, since clinically the vascular permeability is reversed in 24-48 hours, that concentrations would decline fast if histamine was an important mediator, and we therefore conclude that it is unlikely that it is an important mediator of the increase in systemic vascular permeability that is seen early after burns.

The notion that urinary concentrations of methylhistamine were not affected at all is important, because histamine is rapidly converted to methylhistamine in plasma. A true increase in the concentration of histamine in plasma would be paralleled by an increase in that of methylhistamine. As this was not seen, we conclude that some of the histamine found in urine after burns may originate from de novo production in the kidney, which has been shown to occur in other contexts [124].

It is important to note that study V did not address the issue of local vascular permeability in burned skin, in which histamine also may play a part, as indicated by one of the animal studies [63].
4.12 Limitations

The studies in this thesis are all observational. This is the most important limitation, and it precludes detection of definitive causal relations. All the studies were relatively small, which limits their power. They are all single-centre studies, which limits the possibility of generalising the results. That they are single centre studies is also a strength, because exploratory studies like these that aim to describe how things are, may benefit from strict protocols applied for every case. The patients studied were all from selected groups and were severely injured. This also precludes generalisation of the results to other, more mixed, series with some healthier patients as well.

4.13 The future

There are a few experimental studies in animals about the role of HBP in sepsis but none about the role of HBP after trauma or burns. To further test the hypotheses and questions raised by this thesis, it would be interesting to simply block the effects of HBP in a model of ARDS and increased vascular permeability after trauma or burns. HBP is characterised only in humans and pigs. Several models of trauma and hypovolaemic chock have been developed in pigs. It is reasonable to assume that they may include granulocyte activation and release of HBP, with their possible contribution to ARDS and maybe to MOF. There is a blocking antibody active against porcine HBP that may affect the outcome in such a model. The amount of antibody that has to be given to a pig, however, makes such studies expensive.

To block the effects of HBP in patients in critical care would indeed be advanced, as its antimicrobial effect makes it possible that there are beneficial effects in sepsis, and this is supported by an animal study [125].

All the studies in this thesis are hampered by small numbers of patients and by the inclusion of only selected series of seriously ill patients. It would be interesting to find out if concentrations of HBP in plasma have a predictive value in the detection of the development of ARDS or MOF in a larger and mixed group in an ICU. If this is true, it would supply us with a tool to select patients at risk of ARDS who may benefit from early increased ventilator support with low tidal volumes and PEEP to decrease the risk of the development of ARDS.
4.14 Conclusions

1. There is an early rise of CD11b expression on granulocytes after burns and this may explain the large and fast decrease in the granulocyte count as it may promote extravasation in case of firm adhesion. There is a reduction in the CD16 expression on granulocytes after a burn, and this probably explains why IgG-mediated granulocyte phagocytosis is decreased.

2. There is no correlation between plasma concentrations of HBP and increased pulmonary vascular permeability or a decreased PaO$_2$: FiO$_2$ ratio after burns, so it is unlikely that the systemic plasma concentration of HBP mediates these effects.

3. There is an early and predictable change in WBC after a burn. The degree of early decrease of WBC is, independently of TBSA\%, correlated with an increase in pulmonary vascular permeability and with reduced PaO$_2$: FiO$_2$.

4. Immature forms of granulocytes appear in the circulation after burns, so a causal relation with the immunosuppression seen after burns is plausible.

5. Increased systemic plasma concentrations of HBP may predict the development of ARDS after severe mechanical trauma.

6. It is unlikely that histamine plays a dominant part in the systemic increase in vascular permeability after burns.
Environmental impact

As with all human activities, the work reported in this thesis has had an impact on our environment. I have read and written almost everything on screen to minimise the use of paper. Some references have been printed out, and there are still other circumstances under which I have had to handle paper: my estimate is that I have used < 10 kg. The projects themselves include sending, freezing, and analysing of samples, which consume energy but the amount is hard to estimate. Because half the work has been done since I moved from Linköping to Östersund, I have had to travel to attend courses, seminars, and occasionally to meet my supervisor in Linköping and my cosupervisor in Stockholm. Four of these were made by air and the rest by train (and taxi to and from the trainstation). According to the environmental policy of SJ, the electricity used by SJ is totally produced by wind or water-based power [126], which limits the emissions of carbon dioxide and the impact on global warming. I made two return flights from Östersund to Umeå, which added up to an emission of 300 kg carbon dioxide (including shared taxi transfer), according to the travel agent who booked the flight. I made one return flight to Stockholm, calculated by the calculator on SJ’s web page [126] to emit 160 kg CO₂. I made one return flight from Stockholm to Vienna that, according to the same CO₂ calculator, emitted 564 kg CO₂. The total travel adds up to almost one ton of CO₂ for the work with the thesis.
Acknowledgements

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