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Alteration of leucocyte count correlates with increased pulmonary vascular permeability and decreased PaO₂:FiO₂-ratio early after major burns

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Conflict of interests: The company Hansa Medical has filed a patent application on the use of HBP as a diagnostic tool in sepsis. HH is listed as inventor. None of the other authors have any conflicts of interests.
Alteration of leucocyte count correlates with increased pulmonary vascular permeability and decreased $\text{Pa}_2:\text{Fi}_2$-ratio early after major burns
Abstract

Leucocytes are activated systemically and their numbers increase soon after a burn followed by a rapid decline to low normal or subnormal levels, possibly by increased extravasation. Experimental data supports that an important target for such extravasation is the lungs and that leucocytes when they adhere to endothelial cells cause an increase in vascular permeability. We investigated a possible relation between early increased pulmonary vascular permeability or a decreased PaO₂:FiO₂-ratio and the dynamic change in concentration of blood leucocytes after a burn.

This is a prospective, exploratory, single-centre study. We measured the dynamic changes of leucocytes in blood starting early after the burn, pulmonary vascular permeability index (PVPI) by thermodilution, and PaO₂:FiO₂-ratios in 20 patients during the first 21 days after a major burn (>20% total burn surface area %).

Median total burn surface area was 40% (IQR 25-52) and full thickness burn 28% (IQR 2-39). There was a correlation between the early (<24 hours) alteration in white blood cell count and both early increased pulmonary vascular permeability (r=0.63, p=0.004) and the decreased oxygenation index defined as PaO₂:FiO₂<27kPa (p=0.004).

We have documented a correlation between dynamic change of blood leucocytes and pulmonary failure early after burns.

Keywords: azurocidin, burn, HBP, leukocyte, PMN
Introduction

There is increasing evidence that the lung is affected soon after a major burn not only by inhalation injury but also by a condition that simulates acute respiratory distress syndrome (ARDS) with, for example, increased pulmonary vascular permeability and decreased PaO2:FiO2 ratio. The exact mechanisms underlying these findings have, however not been fully elucidated. Earlier investigations and textbooks have proposed histamine, serotonin, and oxygen radicals as possible mediators of this effect in the systemic circulation [1-2], but to our knowledge there are no studies of burns in humans that have set out to investigate the genesis of these early reactions after a burn in which the pulmonary changes have been measured with thermodilution.

Polymorphonuclear cells sampled from burned patients are systemically activated [3-5] and the total leucocyte count is increased within a few hours after a burn but return to low normal or subnormal levels within 24 (-48) hours [6-8]. The impressively large and fast changes in white blood cell (WBC) count after burns may reflect an early release of leucocytes from the bone marrow and subsequent trapping of leucocytes in the vessels of the lung and other vascular beds. In an earlier study we examined ARDS and inhalation injury during the first week after a burn and found a correlation between changes in the WBC count and the development of respiratory failure, as measured by the Murray lung injury score [9], during this period [6]. A similar effect has also been reported in studies of trauma other than burns [10-11]. It is in line with findings of large amounts of extravasated leucocytes in ex vivo pulmonary and other organ preparations from animal models of burns [12-14].

ARDS is characterised by leakage of protein-rich fluid from lung vessels and recruitment of PMN into lung interstitial tissue and alveoli [15]. It is evident from numerous experimental and clinical studies that leucocytes, and PMN in particular, are critically involved in the pathogenesis of ARDS [15-17].

In preclinical investigations PMN have been shown to increase vascular permeability when they adhere to endothelial cells [18], and release of heparin binding protein (HBP, also known as CAP-37
or azurocidin) contributes to this effect [19]. We have, in a pilot study of 10 burned patients, shown that plasma concentrations of HBP are increased early after a moderate to major burn [20].

Earlier studies have shown increased numbers of immature PMN in blood after trauma other than burns [11], and this has, to our knowledge not been investigated previously after burns. If an increased level of immature blood PMN is found after burns, it could explain some of the defective functions of PMN found after burns.

We aimed to study in detail the possible association between alterations in concentration of leucocytes in the blood with the early development (<24 hours) of the increased pulmonary vascular permeability index (PVPI), and with changes in the PaO2:FiO2 ratio. We also measured the concentrations of HBP in plasma and of immature PMN in blood.

**Methods**

This is a prospective, descriptive, exploratory study at a national burn centre. The Regional Ethics Review Board in Linköping approved the study. Patients or their next of kin gave informed consent.

During a two-year period (2009-2011) consecutive adult patients (18 years old and over) admitted to the national burn centre (Linköping University Hospital Burn Unit) with burns involving 20% or more of the total body surface area (TBSA%) were included. Only patients for whom blood could be sampled within 24 hours of the burn were included.

Patients were treated in accordance with a protocol described earlier [21], including the Parkland formula for early resuscitation, ventilator treatment when needed, early enteral nutrition, and early excision and grafting of the burn wound, starting within 1-2 days.

Samples of blood and plasma were taken at the time of inclusion in the study and then at 8, 16, and 24 hours after the burn. After that samples were taken at 06:00, 14:00 and 22:00 on days 2 and 3 after the burn. On days 4, 5, and 6 the sampling time was 06:00 every day and then at 06:00 on days 9, 12, 15, 18, and 21 after the burn. Blood samples were drawn from the arterial catheter for differential WBC count and measurements of blood gases. Blood was also immediately spun to plasma which was stored
in -80° C for subsequent analysis of concentrations of HBP [22]. The highest value within 24 hours (HBP_{max24}) was used to test associations with other variables.

Extravascular lung water (EVLW) and intrathoracic blood volume (ITBV) were assessed with transpulmonary thermodilution (PiCCO®, Pulsion Medical Systems, Munich, Germany) at the same time as blood samples were taken. As proposed in earlier studies [23] we used EVLW:ITBV as a measure of vascular permeability in the lung, the so-called pulmonary vascular permeability index (PVPI) [24]. ITBV is used in the numerator to account for differences in body size and differences in preload. For comparison with other variables, we used the highest PVPI within 24 hours of the burn, PVPI_{max24}.

Alterations in the WBC count and PMN count were assessed as the maximal concentration minus the minimal concentration within 24 hours of the burn (WBCΔ24 and PMNΔ24).

Statistical analysis

Linear regression was used to assess possible associations between two continuous variables. The significance of differences between groups was assessed with the Mann-Whitney U test and the significance of differences in proportions was assessed using Fisher’s exact test. To elucidate the relation between the different variables that are suspected to cause the increased PVPI_{max24} further, we did a multiple linear regression with the two independent variables of interest, TBSA%, and WBCΔ24. Data were analysed with the help of Statistica 9 (StatSoft Inc, Tulsa, OK, USA).

Results

35 patients with TBSA% > 20 were cared for in the unit during the study time. Four were below 18 years of age, one had not suffered a burn but rather had a severe skin condition, six could not be included within 24 hours of the burn, two were not considered in need of intensive care despite burns > 20% and therefore did not receive the catheters needed for sampling and measurement. Thus 22 patients were included in the study. An early decision not to resuscitate two of the patients was taken because of the severity of their injuries, and they both died within 30 hours of the burn and were
excluded from further analysis. Median TBSA% was 40% (IQR 25-52), median full thickness burn (FTB%) was 28% (IQR 2-39), median age was 43 years (IQR 27-63), and three were women. Two patients died within 30 days and four died in the ICU (the two patients that died very early are excluded from these numbers) (Table 1). All patients in the study were treated in a ventilator because of their injuries, not only for surgical revisions.

An inability of the lungs to oxygenate the blood is measured by the ratio of the partial pressure of oxygen, the PaO₂, to the fraction of inspired O₂, the FiO₂. Of the 20 patients who survived more than 30 hours, 6 had their worst PaO₂:FiO₂ < 27 kPa within 24 hours (Table 2).

There were initial rises in WBC count and PMN count, followed by decreases (Figs. 1 a and b). Immature forms of PMN (metamyelocytes, myelocytes, and band forms) were apparent in the circulation after burns and more so in the later phase (> 5 days) (Fig. 2). Reference is 0. The profile of HBP over time is shown in Fig. 1 c. There was no early increase of HBP and after 48 hours the concentrations were stabilised around 15 ng/ml.

There were problems with the thermodilution in patient 10 (inconsistencies in consecutive measurements) and his data was excluded from further analysis in cases where the values from thermodilution was to be used.

WBCΔ24 and PMNΔ24 were significantly correlated with PVPImax24 (p=0.0008 and 0.0007, Fig. 3) and with the worst PaO₂:FiO₂ ratio < 27 kPa within 24 hours (p=0.004 and 0.003, Fig. 4, Table 2) whereas we found no correlation between HBPmax24 and PVPImax24 (p=0.31) or with the worst PaO₂:FiO₂ ratio < 27 kPa within 24 hours (p=1.0) (Figs. 3 and 4, and Table 2).

The result from the multiple linear regression showed that WBCΔ24 was an independent significant explanatory variable (r=0.66; p=0.008) for the PVPImax24, while TBSA% was not (Table 3). The model was accurate in predicting the PVPImax24 (adjusted R²=0.593, p=0.01).
Discussion

We found a clear association between PVPI\textsubscript{max24} and the early alterations in WBC count and PMN count.

Although paralleled by similar studies in trauma from other causes [10-11], and one earlier study from our group on burns [6], this study is to our knowledge the first to show it with the actual measured PVPI and with a high temporal resolution for the dynamics of WBC count during the important first 24-hour period. By looking at these phenomena in the timeframe within 24 hours it is reasonable to believe that the changes in measured variables are induced by the burn itself (not by the subsequent surgical procedures and not from infections). As can be seen from table 1, there were only two patients (no 4 and no 15) that had surgery within 24 hours from the injury. Their respective revisions started 22 and 20 hours after the injury and hence it is unlikely that the revisions have had any important impact on our results. There was an association of WBC\textsubscript{Δ24} with TBSA\% (data not shown) and this also suggests that the rise and fall, within 24 hours of the burn, of WBC count is a direct consequence of the burn.

As TBSA\% is the measure of the size of the injury, it is expected to correlate with outcome variables such as PVPI\textsubscript{max24} or decreased Pa\textsubscript{O2}:Fi\textsubscript{O2} ratio. This was also the case in a simple univariate linear regression and is not surprising (data not shown). What’s new and somewhat surprising in our results is that when we introduced the variable WBC\textsubscript{A24} in a multiple linear regression, the result was that WBC\textsubscript{A24} was the only independent variable to explain PVPI\textsubscript{max24} of the two tested variables. To our knowledge, there is no other biomarker that can accurately, and independently from TBSA \%, sort out the patients that develop respiratory failure so early after burns. C-reactive protein for example, is often used as a marker of inflammation but in burns it reacts slowly and reaches its peak not until five to seven days after the burn. We tested for correlations of C-reactive protein with the measured pulmonary variables in our material but found none at all, neither early nor late (not shown).

We also tested the possible relation of the maximal absolute number within 24 h of WBC with the measures of pulmonary failure and found such correlations, albeit markedly weaker than for WBC\textsubscript{A24},
suggesting that it is the *decrease* of WBC that is most important for development of respiratory failure.

For severe non burn trauma there are studies showing that plasma levels of certain cytokines had the ability to early discriminate between patients developing or not developing ARDS [25] but another study showed that this was not the case [26].

The analysis of WBC count is fast, easy, cheap, and readily available. Our results further show that the two different measures of early leucocyte (WBC count or PMN count) dynamics give similar information which facilitates their use.

ARDS is intimately associated with increased pulmonary vascular permeability and increased accumulations of extravascular fluid in the lungs. This leads to decreased oxygenation (decreased P:F ratio), which is the main clinical feature of ARDS. We were interested in factors that explained the early respiratory failure after burns and chose to look at the increased PVPI$_{\text{max}24}$ and the decreased $\text{PaO}_2:\text{FiO}_2$ as endpoints. WBC$_{\Delta24}$ correlates with both endpoints, which strengthens the results.

A definitive diagnosis of ARDS was not possible within 24 hours because we had no chest radiographs. The limit chosen in our study, P:F = 27kPa, is the limit for ARDS according to the old criteria, and the limit for moderate or severe ARDS in the new criteria [27].

We sought to find an association between plasma concentrations of HBP and vascular permeability of the lungs measured by PVPI. As no such correlation was seen it indicates that systemic concentrations of HBP do not seem to act directly in an endocrine fashion to mediate the early increased vascular permeability that follows a burn. It may however be that HBP, when it is released from PMN that are adherent to the endothelium, acts locally in a paracrine fashion and that the concentrations reached in the systemic circulation are insufficient to activate the endothelium.

In our earlier pilot study [20] we found considerably increased concentrations of HPB in plasma soon after burns. In the present study, although we drew the samples more often and sooner after the burn to try and describe the dynamics of HPB in plasma better, we were unable to find the previously reported
early increase in HBP. The concentrations during the later phase of the study were also higher in the present study. It is not clear why the results in the present study differed from those in the pilot study. Limitations of the study include sample size (n=22) and the fact that inhalation injury was not described in the protocol. Even if inhalation injury has contributed to the changes in WBC count, increases in PVPI, and reductions in $\text{PaO}_2:\text{FiO}_2$, this fact does not change the utility of the results. Another limitation is that the changes in leucocytes happened earlier and more rapidly than expected, and a chest radiograph was not obtained within 24 hours in the protocol, making a definitive diagnosis of ARDS impossible at this point. However, most patients who develop early problems with oxygenation have ventilator support with appropriate PEEP adjustments to open up the lung before a radiograph may be taken. This may in a sense treat the ARDS (the decreased $\text{PaO}_2:\text{FiO}_2$ and possibly the radiograph), but the pathophysiological disturbance (the increased PVPI) may still be there and is correctly measured by transpulmonary thermodilution. 

In our series immature forms of PMN appeared after the burns (Fig 2). This corroborates earlier findings in trauma not caused by burns [11] but has not been shown previously in human burns. The reason may be that a large proportion of newly-matured PMN are released from the bone marrow after the burn and many extravasate early. Release of new mature PMN may be hampered by the fact that the normal maturation process is slow and also by a suggested suppression of the bone marrow induced by the burn [8, 28]. The secretory vesicles are lacking in immature forms of PMN because they are the last to form in the maturation process [29]. This may be of clinical importance, because fusion of secretory vesicles with the cellular membrane is part of the process by which adhesion molecules are upregulated at the time of activation of PMN [29]. This state of immature cell granulocytopenia after the burn may explain the fact that the oxidative burst of PMN, chemotaxis, and Ig-mediated phagocytosis are suppressed after a major burn [4, 30-32], factors that may all contribute to immunosuppression after a burn.

Regarding the leucocytes that are really trapped in the pulmonary circulation, and subsequently migrate to the alveoli, Davis et al found marked immune hyporesponsiveness and decreased number of leucocytes in alveoli among nonsurvivors by means of bronchoalveolar lavage early after burns in
patients with suspected inhalation injury [33]. Hence it seems that the accumulation of immunocompetent leucocytes in the alveoli is either important in itself or a marker for some other factor related to mortality. Another very recent study on (non-burn) ARDS [34] propose that the retention of activated leucocytes in pulmonary vessels is important to protect the host from the systemic action of these leucocytes. This model may fit with the observation of decreased amount of and decreased level of activation of leucocytes in alveoli of burn nonsurvivors. Unfortunately our study is too small for a specific look at the nonsurvivor group.

Davis and colleagues also investigated the role of inhalation injury on systemic levels of different cytokines and its impact on mortality after burns [35]. Their findings support that the inhalation injury affects both mortality and the systemic inflammation in a graded manner. Our result implies that $WBC_{Δ24}$ is the most important factor for pulmonary failure but unfortunately, with the current ethical approval and study protocol, we could not control for inhalation injury. Inhalation injury may have a profound effect on both the sequestration of leucocytes in the pulmonary circulation and on pulmonary vascular permeability, either by the local epithelial injury itself or mediated by extravasating leucocytes. It is possible that concurrent inhalation injury in our patients have accounted for some degree of $WBC_{Δ24}$ and also to the measured $PVPI_{max24}$. A piece of indirect evidence of the patient’s inability to increase the number of leucocytes after a burn is that these patients undergo massive surgical tissue trauma in terms of revision and skin grafting during the initial week after the burn. In a patient with normally functioning bone marrow this would likely lead to leucocytosis, which we did not see in our series (Fig. 1).

Our results indicate that a $WBC_{Δ24}$ greater than 10 suggests high probability of concurrent pulmonary failure and hence that noninvasive ventilation probably would be futile in such cases. It is important to remember that this study was not designed to investigate this specific question and that such a clinical recommendation would require further and larger studies.
**Conclusion**

Maximum early dynamic change in WBC count (WBC$_{Δ24}$) was significantly associated with early increased pulmonary vascular permeability, and significantly associated with an early decreased Pa$_{O_2}$:Fi$_{O_2}$ ratio.

**List of abbreviations**

- **ARDS** Acute respiratory distress syndrome
- **CAP-37** Cationic antimicrobial protein of 37kD (another name for HBP)
- **EVLW** Extravascular lung water
- **FTB %** Full thickness burn
- **HBP** Heparin binding protein
- **ICU** Intensive care unit
- **ITBV** Intrathoracic blood volume
- **P:F** Arterial partial pressure of oxygen divided by fraction of inspired O$_2$ (P$_a$O$_2$:F$_i$O$_2$)
- **PiCCO** Pulse-induced contour cardiac output
- **PMN** Polymorphonuclear leucocyte
- **PVPI** Pulmonary vascular permeability index
- **TBSA %** Total burn surface area
- **WBC** White blood cell
Acknowledgements:  Lars Söderström for statistical advice.

References


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Data presented as %, numbers, median, quartile range as appropriate.
Table 2. Results in relation to decreased oxygenation.

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</table>

Count, median (interquartile range), percent as appropriate.
Table 3. Parameters contributing to PVPI_{max 24} in a multiple linear regression

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coefficient of regression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC_{Δ24}</td>
<td>0.66</td>
<td>0.008</td>
</tr>
<tr>
<td>TBSA%</td>
<td>0.20</td>
<td>0.348</td>
</tr>
</tbody>
</table>

HBP, Heparin Binding Protein; WBC, White Blood Cell count; TBSA%, Total Burn Surface Area. The model showed a high accuracy to predict PVPI_{max 24} (adjusted $R^2=0.553$, p<0.003).
Fig 1
Fig 2
Fig 3
Fig 4
Legends:

**Fig. 1** Dynamic change of white blood cell count (WBC), polymorphonuclear leucocyte (PMN) count and plasma heparin binding protein (HBP). Note the change of scale after 72 hours. Square and box indicate the median and interquartile range respectively.

**Fig. 2** Immature forms of polymorphonuclear leucocytes (PMN). Note the change of scale after 72 hours. Square and box indicate the median and interquartile range respectively, circles indicate outliers.

**Fig. 3 a, b and c** PVPI\textsubscript{max24} was significantly associated to WBC\textsubscript{Δ24} (r=0.77 p<0.001) and to PMN\textsubscript{Δ24} (r=0.76 p=0.001) but not to HBP\textsubscript{max24} (r=0.28 p=0.324). (WBC, White Blood Cell Count; PMN, Polymorphonuclear leucocytes; HBP, Heparin binding protein).

**Fig. 4 a, b and c** The relation of WBC\textsubscript{Δ24} and of PMN\textsubscript{Δ24} with presence of Pa\textsubscript{O2}:Fi\textsubscript{O2} < 27 kPa within 24 hours. The relation was significant (p=0.004 and 0.003). There was no relation of HBP\textsubscript{max24} with Pa\textsubscript{O2}:Fi\textsubscript{O2} < 27 kPa (p=1.0). (WBC, White Blood Cell; PMN, Polymorphonuclear leucocytes; HBP, Heparin binding protein). Square and box indicate the median and interquartile range respectively.