WOUND HEALING AND SKIN IN SEVERE SEPSIS

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Abstract

It is a generally accepted dogma that sepsis disturbs skin function and wound healing, but surprisingly there is only remote pathophysiological evidence available behind that presumption. As the skin is the largest defensive barrier, the skin dysfunction in severe sepsis deserves more attention.

In this study, the suction blister model was used to create experimental wounds. The study population included 44 patients with severe sepsis and 15 controls. The blister fluid was collected to analyse cytokine profile of the skin. The transepidermal water loss and blood flow from the wound were measured. A 4mm biopsy was taken under local anaesthesia on the first and the eighth day of the study from the healthy looking skin. Then 15 healing suction blisters were excised. Serum samples were also collected on the first day of the study.

The barrier restoration was diminished, and the inflammation in the wound was more intense in severe sepsis than in the controls. The expression of the basement membrane components Laminin-332 and type IV collagen decreased during the septic disease, but increased over the next 3 months without achieving the level of the controls. The expression of tight junction proteins remained nearly intact in the healing wound in severe sepsis compared to the controls. The expression of occludin on the leading edge of the migrating keratinocytes was more restricted and late in severe sepsis compared to the controls. The levels of the tumour necrosis factor (TNF), interleukin-10 (IL-10) and IL-6 in skin blister fluid were higher in the sepsis compared to controls. The blister fluid and serum cytokine response in the sepsis differed since the levels of epidermal growth factor, vascular endothelial growth factor, TNF and basic fibroblastic growth factor (bFGF) in the blister fluid did not correlate with the levels of serum. The septic patients with multiple organ failure had higher levels of several cytokines than patients without organ failure. Survivors had lower levels of IL-10 and bFGF in blister fluid than the non-survivors.

This study offers novel findings for skin and wound healing in sepsis. Together, all the findings suggest that skin dysfunction in severe sepsis exists even when the most profound structures remain intact. Understanding these mechanisms of impaired wound healing can improve future treatments, such as the timing of surgery.

Keywords: basement membrane, cytokine, sepsis, skin, suction blister model, tight junction, wound healing
Tiivistelmä

Sepsiksen ajatellaan heikentävän haavanparanemista, mutta tieteellistä näyttöä on niukasti. Iholla on keskeinen osa elimistön puolustuksessa ja tasapainon ylläpidossa, joten sen toiminnan häiriöyhtymä on toistuvasti tulehdusessa ansaitsee suuremman huomion.

Imurakkulahaavat tehtiin 44 septiselle potilaalle ja 15 kontrollille. Haavoista mitattiin veden haihtumista ja veren virtausta sekä otettiin imurakkulaneste näytteeksi sytokiehitystä varrettiin. Tutkimuksen ensimmäisenä ja kahdeksantena päivänä otettiin 4mm biopsiat terveeltä iholta ja 15 potilaalta poistettiin näytteeksi paraneva imurakkulahaava. Seeruminäytteet otettiin tutkimuksen ensimmäisenä päivänä.

Veden haihtuminen haavalta oli voimakkaampaa eli ihon barrierin palautuminen oli hidastunut septisillä potilailla verrattuna kontrolleihin. Haavassa havaittu tulehdus oli voimakkaampi. Tyvikalvon komponenttien Laminiini-33:in ja tyypin IV kollageenin ilmeneminen oli vähäisempää sepsiksen aikana ja lisääntyi 3kk kohdalla, mutta ei kuitenkaan saavuttanut kontrolleihin tasoja. Tiivisliitosproteiinien ilmeneminen oli lähes muuttumaton sepsiksessä kontrollien verrattuna. Okludiinin ilmeneminen sen sijaan paranevassa haavassa vaeltavien keratinosyyttien etureunassa oli rajoitettuna ja myöhäispää sepsiksessä kuin kontrolleilla. Sytokiehiä tuumorinėkrositekijää (TNF), interleukiini-10 (IL-10) ja IL-6 olivat koholla imurakkulanesteessä verrattuna kontrolleihin. Epidermaalinen kasvutekijä, verisuonten endoteeli-kasvutekijä, TNF ja perusfibroplastinen kasvutekijä (bFGF) pitoisuudet rakkulanesteessä erosiidat sepsikseen. Tiiviitliitokset pitoisuusliitokset eli ihon sytokiehitysmuoto erosi sepsikseen. Potilailla, joilla oli moniepinivaurio, todettiin korkeampia sytokiineisiä (IL-10 ja bFGF) rakkulanesteessä. Tämä tutkimus tarjoaa uutta tietoa ihosta ja haavanparanemisesta sepsiksessä. Tulosten perusteella voidaan todeta, että ihon toimintahäiriö on sepsiksessä todellinen, vaikka kaikkein perustavin voisi tutkintotanko, mutta sepsikseen sekä toimintahäiriöön liittyvat muuttumattomina. Toimintahäiriöön ymmärtäminen voisi auttaa septisen potilaan hoidossa, kuten kirurgisten toimenpiteiden ajoittamisessa paranemisen kannalta mahdollisimman otolliseen aikaan.

Asiaan: haavanparaneminen, iho, imurakkula, sepsis, sytokiehitys, tiiviit liitokset, tyvikalvo

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For Lasse, Matilda and Akseli
Only the love of truth brings about miracles.

Johannes Kepler
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Marjo Koskela
Abbreviations

ACCP American College of Chest Physicians
APACHEII Acute Physiology and Chronic Health Evaluation II
aPKC Atypical protein kinase C
BF Blood flow
bFGF Basic fibroblastic growth factor or FGF-2
CARS Compensatory anti-inflammatory response
CCL2 Monocyte chemotactic protein 2
DIC Disseminated intravascular coagulation
CX3LI Fractalkine
CXCL1 Chemokine (C-X-C motif) ligand 1
CXCL8 Interleukin-8
EGF Epidermal growth factor
FGF Fibroblastic growth factor
HGF Hepatocyte growth factor
ICU Intensive Care Unit
IGF Insulin-like growth factor
IFN Interferon
IL Interleukin
JAM-A Junctional adhesion molecule A
MMP Matrix metalloproteinase
MODS Multiple organ dysfunction syndrome
MOF Multi organ failure
Mupp1 Multiple PDZ domain protein
NISCH Neonatal sclerosing cholangitis associated
NO Nitric oxide
OUH Oulu University Hospital
PDGF Platelet-derived growth factor
PU Pressure ulcer
SD Standard deviation
SIRS Systemic inflammatory response syndrome
SOFA Sequential organ failure assessment
TGFα Transforming growth factor alpha
TGFβ Transforming growth factor beta
TEWL Transepidermal water loss
TIMP Tissue inhibitor of matrix metalloproteinase
<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor (previous TNFα)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>ZO-1</td>
<td>Zonula occludens -1</td>
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List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


* with equal contribution.
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1 Introduction

The most profound task of the body is to maintain homeostasis. Severe sepsis challenges the body to modify the behaviour of cells and organs to keep that balance despite a churning inflammatory storm.

Sepsis, a systemic inflammatory response syndrome (SIRS) evoked by an infection, triggers the production of several mediators that alter the immune response. These mediators include, but are not restricted to, several cytokines and growth factors (Adib-Conquy & Cavaillon 2007). Even though sepsis is dominantly characterized by the prompt production of pro-inflammatory mediators, it is also accompanied by the production of anti-inflammatory mediators (Castellheim et al. 2009). Imbalanced systemic immune responses can lead to the accumulation of leukocytes, disseminated intravascular coagulation (DIC), and microcirculatory dysfunction with subsequent apoptosis and necrosis of parenchymal cells, finally resulting in the development of multiorgan dysfunction syndrome (MODS) and organ failure (Rittirsch, Redl & Huber-Lang 2012).

In recent years, our understanding of the pathophysiology of sepsis has improved due to enhanced intensive care; however, the number of deaths per year remain disturbingly high (Lagu et al. 2012). Sepsis and its various adverse sequelae, such as septic shock and MODS, continue to be among the most common causes of death in the noncoronary intensive care unit (Balk 2000).

Although the skin is the largest organ of the body, it is rarely seen as a vital one like the liver or the heart. The destruction of only 15% of the skin's total body surface area is sufficient to be life threatening, and the total rupture of the barrier function of the skin induces massive water loss that will rapidly cause deadly hypovolemic shock (Blais et al. 2013). Skin is then on the front line when maintaining homeostasis and protecting our body from outer threats. In addition to the physical barrier consisting of the corneal layer barrier and the structure of the epidermis and dermis, the skin is also an immunologic barrier. Antimicrobial peptides, low temperature, and pH, the commensal bacteria, and the corneal layer barrier are all mechanisms that work as a skin surface host defences (Krishna & Miller 2012).

Wound healing is a concrete example of the body’s response to a threat that tries to unbalance this homeostasis. In normal circumstances, to repair injured skin takes only days. This repair includes partly overlapping processes, such as inflammation, angiogenesis, collagen deposition, granulation tissue formation,
epithelization and maturation (Broughton, Janis & Attinger 2006a, Eming, Martin & Tomic-Canic 2014).

The stratum corneum formed by keratinocytes is not formed at the beginning of wound healing, so therefore, the repair of the injured barrier in the healing wound is performed primarily through the basing of tight junction structures (Kallioinen et al. 1995, Lévy et al. 1995). In severe sepsis, tight junctions leak in several organs, but there is no data on the cutaneous tight junctions in sepsis.

In a wound, weak inflammatory response increases the risk of infection, and excessive inflammation contributes to disturbed wound healing (Florin et al. 2006, Wang et al. 2006). A balance between these two extremities has to be established for the wound to heal properly. Systemic infection can have various effects on local levels and need to be studied.

This study investigated the effect of systemic infection, severe sepsis, on skin. The search for clinical strategies that might improve the body's natural repair mechanisms needs to be based on a thorough understanding of the basic biology of repair and regeneration. We wanted to illustrate impaired epidermal wound healing and decipher the focal structures of the skin during severe sepsis. This study’s goal was to increase the understanding of the role of the skin in systemic inflammation, so that in the future, instead of ignoring it’s role we can actively enhance the circumstances of wound healing in situations where it has become diminished.
2 Review of the literature

2.1 Wound healing in general

Wound healing is a complex process that begins immediately upon injury and ends after months of remodelling. The healing process depends on local wound factors, systemic mediators, the underlying disease, and the type of injury (Schreml et al. 2010). Successful wound management requires a thorough understanding of wound healing and the factors that influence it. These factors combine to determine whether acute wound healing is occurring or if an abnormal healing process is leading to a chronic wound. Chronic wounds are a result of an inadequate repair process that cannot restore anatomic and functional integrity within an appropriate length of time. In addition to chronic ulcers where prolonged inflammation and the inability to re-epithelialize enable pathologic wound healing, overgrowth of granulation tissue leads to pyogenic granulomas and an excessive fibrotic response to hypertrophic scars and keloids (Sun et al. 2014). Traditionally, wound healing is divided into three phases: Inflammation, proliferation, and remodelling (Broughton et al. 2006a).

The inflammatory phase begins right after injury, and it is characterized by haemostasis and inflammation (Broughton et al. 2006b) (Fig 1). Injured endothelial cells immediately vasoconstrict and the intrinsic coagulation pathway and haemostasis are activated. The clot that forms is made of collagen, platelets, thrombin and fibrinogen and provides a provisional extracellular matrix for cell migration (Sun et al. 2014). It also serves as a reservoir of growth factors needed during the later stages of the healing process (Werner & Grose 2003). Injured or activated parenchymal cells, coagulation and activated complement pathways generate numerous vasoactive mediators and chemotactic factors that recruit inflammatory cells to the wound (Martin & Leibovich, 2005, Singer & Clark 1999). Within a few minutes, leukocytes arrive, followed by monocytes and lymphocytes (Barrientos et al. 2008). They produce proteinases and reactive oxygen species to defend the wound site from microorganisms; they are the source of important growth factors and cytokines (Table 1) (Werner & Grose 2003).
Fig. 1. Skin wound healing. Phase A Hemostasis can also be considered as part of Phase B Inflammation. PDGF platelet-derived growth factor, TGFβ transforming growth factor beta, CX3CL1 fractalkine, CCL2 monocyte chemotactic protein 2, VEGF vascular endothelial growth factor, EGF epidermal growth factor, IL-1 interleukin 1, TNF tumour necrosis factor, TGFα transforming growth factor alpha, CXCL1 chemokine (C-X-C motif) ligand 1, CXCL8 interleukin-8, FGFs fibroblastic growth factors, VEGF vascular endothelial growth factor, HGF hepatocyte growth factor, IGFs insulin-like growth factor, IFNs interferons, MMPs matrix metalloproteinases, TIMPs tissue inhibitor of matrix metalloproteinases, respectively. (From Sun et al. 2014. Reprinted with permission from the American Association for the Advancement of Science.)
Table 1. Growth factors and cytokines in wound healing.

<table>
<thead>
<tr>
<th>Growth factor or Cytokine</th>
<th>Cells</th>
<th>Function</th>
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<tbody>
<tr>
<td></td>
<td>Ker¹</td>
<td>Fib²</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>EGF</td>
<td>x</td>
</tr>
<tr>
<td>Transforming growth factor</td>
<td>TGF-beta</td>
<td>x</td>
</tr>
<tr>
<td>Platelet derived growth factor</td>
<td>PDGF</td>
<td>x</td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
<td>x</td>
</tr>
<tr>
<td>Tumour necrosis factor</td>
<td>TNF</td>
<td>x</td>
</tr>
<tr>
<td>Interleukin 1</td>
<td>IL-1</td>
<td>x</td>
</tr>
<tr>
<td>Interleukin 6</td>
<td>IL-6</td>
<td>x</td>
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<tr>
<td>Interleukin 10</td>
<td>IL-10</td>
<td>x</td>
</tr>
<tr>
<td>Interleukin 4</td>
<td>IL-4</td>
<td>x</td>
</tr>
<tr>
<td>Fibroblast growth factor</td>
<td>bFGF (KGF, FGF2)</td>
<td>x</td>
</tr>
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</table>

¹ Keratinocytes, ² Fibroblasts, ³ Endothelial cells, ⁴ Macrophages, ⁵ Neutrophils, ⁶ Platelets, ⁷ Other, ⁸ Inflammation, ⁹ Granulation tissue formation, ¹⁰ Reepithelization, ¹¹ Matrix formation and remodelling, ¹² Angiogenesis, ¹³ Scarring, ¹⁴ Macrophage infiltration, ¹⁵ Neutrophil infiltration
Usually 48 to 96 hours after injury, monocytes in the nearby tissue and blood are recruited to the area and transformed into macrophages (Broughton et al. 2006a). Activation of the macrophages is critical and important for the transition into the proliferative phase. Activated macrophages mediate angiogenesis and fibroplasia by synthesizing certain growth factors (see Table 1) (Fig 1) (Barrientos et al. 2008, Werner & Grose 2003). Matrix metalloproteinases (MMPs), expressed by keratinocytes, fibroblasts, monocytes, and macrophages in response to TNF, clear the inflammatory debris and enable the migration of individual wound cells through the extracellular matrix (Broughton et al. 2006a). Remodelling or the maturation phase starts when the apoptotic and proteolytic mechanisms remove the excess matrix along with synthesis. This phase involves a transition of the dermis from type III to type I collagen predominance in addition to collagen remodelling and altered collagen synthesis that results in a scar (Gill & Parks 2008). As a result, the tissue structure and function of the skin is restored to the best of its ability; the tensile strength of the wounded skin regains around 40% of its original strength at 1 month and around 70% by 1 year (Sun et al. 2014).

2.2 Cytokines and altered wound healing

Several cytokines are present at the wound site. The dynamic expression with temporal and spatial characteristics manifests the regulation, and important alterations at the levels of one factor that eventually affect the production of other cytokines (João De Masi et al. 2016). Many of the cytokines can have either a beneficial or a detrimental role on wound healing depending on dose and timing.

Prolonged exposure of proinflammatory cytokines to wound tissues may affect the wound healing and disturb it (McCarty & Percival 2013). Chronic exposure of skin cells to TNF and IL-1 is a contributing factor in connective tissue disease where the levels of TNF and IL-1 are shown to be elevated in the fluids of chronic wounds (McCarty & Percival 2013). Increased levels of IL-6 have been associated with several skin pathologies, such as psoriasis, and IL-6 knockout mice have demonstrated delayed acute wound healing (Wang et al. 2004, Gallucci et al. 2000). Daily applications of TNF can show an inhibitory effect on tissue repair in rats (Rapala 1996, Rapala et al. 1991). Exogenous application of TGFbeta has been shown to be a potent stimulator of granulation tissue whereas dysregulated overproduction contributes to tissue fibrosis (Schmid et al. 1998).

In rats, EGF and VEGF, IGF and bFGF seem to accelerate healing and stimulate angiogenesis, collagen maturation and fibroplasia in acute wounds (De
Masi et al. 2016). Application of EGF to the healing wounds of rats can enhance wound healing by increasing fibroblast proliferation without collagen overproduction (Laato et al. 1987). IL-10 overexpression reduces scar formation in a dose-dependent manner in murine wounds and decreases the inflammatory response in that wound (King et al. 2014). An abnormal expression of bFGF is reported in animal models with delayed wound healing and exogenous bFGF has been successfully used to improve cutaneous wound healing (Demidova-Rice et al. 2012, Matsumoto et al. 2013). Clinical trials have also showed only modest improvements in healing rates (Barrientos et al. 2008). The levels of VEGF are low in wounds that exhibit delayed healing, while exogenous VEGF accelerates healing in many animal studies, Yet human studies seem to have failed to repeat these results (Johnson & Wilgus 2014). IL-4 overexpression in mice resulted in delayed wound closure, decreased tensile strength and increased neutrophil infiltration that might indeed contribute to the impaired wound healing (Zhao et al. 2016).

Bacterial components may contribute to impaired repair mechanisms of the host by interfering with cell-matrix interactions’ attenuating the inflammatory response. Inflammation in wound healing is also important for the removal of the micro-organisms contaminating the wound area (Guo & Dipietro 2010). Bacteria and endotoxins elongate the inflammatory phase by prolonging the elevation of pro-inflammatory cytokines, such as IL-1 and TNF (Edwards & Harding 2004).

Glucocorticoids exert a deleterious effect on wound healing by interfering with inflammation, fibroblast proliferation, collagen metabolism, angiogenesis, connective tissue metabolism, wound contraction, and re-epithelization (Anstead 1998). The effect of glucocorticoids on wound healing is dose-dependent. In rats, exogenous dexametasoned reduced tensile strength in low doses and prevented healing completely in larger doses (Fenton &West 1963). Dexametasoned also interferes with the synthesis and degradation of Type I and Type III collagen (Oishi et al. 2002). Many of the actions of glucocorticoids are mediated by influencing the expression of cytokines like TNF, KGF, TGFbeta, and IL-1 (Beer et al. 2000). Glucocorticoid-induced immunosuppression in mice resulted in delayed wound healing, while IL-6 treatments augmented this effect (Gallucci 2001). In sepsis, glucocorticoids reduce the transcription of proinflammatory genes, resulting in an important immunosuppressive effect. A low dose of hydrocortisone therapy is widely used in septic shock to inhibit inflammation but it is not always effective in suppressing the cytokine storm that is driven by a systemic inflammation (Martin-Loeches et al. 2015). Compared to hydrocortisone, dexamethasone is more long
lasting, has higher anti-inflammatory effects, lower mineralocorticoidal effects, and
does not interfere with the water balance (Cicarelli et al. 2007).

2.3 Skin as a barrier

The skin protects the body from unwanted influences from the environment as well
as excessive water loss. It forms an outside-in and also an inside-out barrier using
two different mechanisms. While in simple epithelia tight junctions (TJ) form the
main (paracellular) barrier, in the skin the stratum corneum also plays an important
role in barrier function. Wounding always disrupts the epidermal barrier of the skin
and an important part of that healing is restoration of the epidermal barrier function
to prevent the loss of water and solutes.

2.3.1 The Stratum corneum barrier

The Stratum corneum barrier or primary barrier consists of corneocytes, that is,
dead cells, and intercellular lipids (Menon et al. 2012). It is the uppermost layer of
the skin and forms a very important part of the skin barrier. For decades it was
thought to be the only barrier present in the epidermis (Kirschner & Brandner 2012).
The stratum corneum barrier leaks during several skin diseases, such as atopic
dermatitis, leading to both scaling and increased water loss (Marks 2004).

2.3.2 Tight junctions

The secondary barrier in the epidermis is formed by tight junction structures. Tight
junctions form a collar around each cell just beneath the cell surface where they
seal the intracellular space and prevent penetration of the luminal content between
the lining cells (Van Itallie & Anderson 2014). Each tight junction forms a zonule
(a continuous circumferential band) around the cell. These are seen as “kissing
points” in the stratum granulosum, whereas electron microscopy reveals structures
containing TJ proteins, but does show the typical morphology in all layers in
epidermis except for the stratum basale (Fig 2) (Brandner et al. 2002). This
structure reflects the complexity of the epidermis. Claudin-1 is seen in all viable
epidermal layers, occludin is restricted to the stratum granulosum, and ZO-1 is seen
in the upper layers of the stratum spinosum in addition to the stratum granulosum,
while the stratum corneum is always negative (Fig 2) (Brandner 2009, Brandner et al.
2002). Several TJ proteins have also been identified in human keratinocytes, namely,
claudin-1, claudin-4, claudin-5, claudin-7, JAM-A (junctional adhesion molecule A), cingulin, ZO-1, Mupp1 (multiple PDZ domain protein) and aPKC (atypical protein kinase C) (Brandner et al. 2006). Also claudin-8 and claudin-17 have been identified on the mRNA level in keratinocytes.

Fig. 2. The distribution of tight junctions in the skin. The layers of epidermis and dermis are marked here as SC = stratum corneum, SG = stratum granulosum, SS = stratum spinosum, SB = stratum basale, LL = lamina lucida, LD = lamina densa, BM = basement membrane and D = dermis.

Generally, tight junctions form a selective barrier that controls the paracellular movement of molecules down the electrochemical gradients. The permeability of TJs depends on their composition (Günzel & Yu 2013). Several endogenous and exogenous substances, for example growth factors, cytokines and bacterial toxins, affect the permeability of the TJ. The composition of a TJ depends on cell type and state of differentiation, and on the physiological and pathological stimuli (Brandner et al. 2015).
The crucial role of claudins are supported by the finding, that in claudin-1 deficient mice, the epidermal barrier is severely affected leading to dehydration, wrinkled skin, and death of the animals within 1 day of birth. Mutations in the gene coding claudin-1, which results in a loss of the protein, have been identified to be responsible for the neonatal sclerosing cholangitis associated with ichthyosis (NISCH syndrome) (Hadj-Rabia et al. 2004). This is so far the only tight junction-related skin disease, even though several other skin diseases lead to alterations of the tight junction proteins and localization (Brandner et al. 2008). In psoriasis, occludin, ZO-1 and claudin-4, expression was broadened when compared to normally restricted expression (Kirschner et al. 2009, Peltonen et al. 2007).

The same effect was also observed if the skin was colonised with pathogenic bacteria (Ohnemus et al. 2008). In squamous cell carcinoma, occludin and claudin-4 were found in keratinized areas that do not border the lumina of body surfaces, suggesting that they may separate tumour areas from external influences, such as chemotherapeutical drugs and the immune system (Brandner et al. 2008). The combination of occludin and claudin-1 seems to be needed for the establishment of an effective paracellular barrier (Furuse et al. 2002). Occludin positive and claudin-1 deficient skin layers allow the passage of paracellular tracers. Further, claudin-6 expression in epidermis may have a relationship with the unstable temperature control and dehydration frequently observed in premature infants (Troy et al. 2009, Troy, Rahbar et al. 2005, Kursad Turksen & Troy 2002). Over-expression of the mutant forms of occludin leads to changes in the function of the TJs (Bamforth et al. 1999). The role of occludin is, however, still unclear, because occludin knock-out mice displayed well developed TJs (Saitou et al. 2000).

TJ proteins are also found outside the distinct membrane structures, suggesting that they have also membrane structure independent functions (Kirschner & Brandner 2012). Recent findings suggest that TJs play a role not only in the epidermal barrier function but also in both the epidermal differentiation and the stratum corneum barrier function (Kuroda et al. 2010, Sugawara et al. 2013).

2.3.3 The Basement membrane

An intact basement membrane at the dermal-epidermal junction is essential to the viability of the skin. It determines the polarity of the epidermis and provides a barrier to epidermal migration (Böhnert et al. 1986). The basement membrane zone is divided into hemidesmosomes, lamina lucida, lamina densa, and anchoring fibrils (Fig 2) (Borradori & Sonnenberg 1999). Anchoring filaments connect...
hemidesmosomes to lamina lucida. Laminin-332 is the main constituent of the
anchoring filaments, but it is also expressed in lamina densa (Fig 2) (Masunaga
membrane to the dermis, are connected to the laminin-332 and type IV collagen in
the basement membrane. Recent studies show that type IV collagen and laminin-
332 are produced by the epidermal keratinocytes and dermal fibroblasts (El

The epidermis and the dermis never function independently (Finch et al. 1989,
1998). Past studies have shown that laminin-332 is essential to epidermal
attachment (Pulkkinen et al. 1994). Laminin-332 forms a covalent complex with
laminin-6 or -7, and this complex interacts with type IV collagen in the basement
membrane through nidogen (Champliaud et al. 1996). Laminin-332 plays a crucial
role in facilitating the dermal-epidermal adhesion in normal skin and it has been
demonstrated that the exogenous application of laminin-332 improves
transplantation of the keratinocyte attachment to the granulation tissue in wound

Genetic abnormality of laminin-332 leads to epidermolysis bullosa (Pulkkinen
& Uitto 1999, Ryan et al. 1996). The development of autoantibodies to laminin-
332 leads to an acquired autoimmune blistering disease (Domloge-Hultsch et al.
1992, Shimizu et al. 1995). In animal models, the antibodies of laminin-332
directly produce dermal-epidermal separation (Lazarova et al. 1996).

Type IV collagen is expressed at the basement membrane in the external root
sheet (Kobayashi et al. 1989, Messenger et al. 1991). It is the major collagenous
component of all basement membranes, and each type IV collagen is a trimer
composed of 3-α chains. In an epidermal basement membrane, α1, α2, α5 and α6
chains are found (Hasegawa et al. 2007). In vitro experiments have shown that type
IV collagen is a highly specific substrate that interacts with the endothelial,
epidermal, and mesenchymal cells. Various linkages between type IV collagen
molecules produce a nonfibrillar polygonal assembly that serves as scaffolding for
the deposition of other matrix glycoproteins and the attachment of cells (Aumailley
et al. 1986, Charonis 1985, Yurchenco 1986). Type IV collagen facilitates cell
attachment to basement membranes via interactions with the α1β1 and α2β1
integrin receptors (Vandenberg et al. 1991). Mutations in genes encoding the type
IV collagen cause Alport syndrome (Kashtan et al. 1999).

Following any injury that penetrates or disrupts the basement membrane, the
epidermal cells lose their contact with the basement membrane and come into
contact with the naked dermis. Under these conditions, the epithelial cells modify their behaviour to cover and close the wound. This process includes the upregulation of proteolytic enzymes and other changes that accompany conversion to a migratory phenotype (Nishiyama et al. 2000). The wounding of the epidermis leads to increased expression and deposition of laminin-332 to repair the BM and migration of the keratinocytes over the exposed dermal collagen followed by epidermal proliferation (Martin 1997, Woodley 1999, Nguyen 2000).

2.4 Skin microcirculation in sepsis

There are three interconnected networks that take care of blood supply of the skin. The suprapapillary plexus runs along the papillary layer of the dermis and gives rise to single loops of capillaries within each dermal papilla. Beneath the suprapapillary plexus is the cutaneous plexus. It lies at the boundary of the papillary and reticular layers of dermis, and the venous blood from the suprapapillary plexus drains into the veins of the cutaneous plexus (Fig 3). The hypodermic or subcutaneous plexus is in the subcutaneous tissue layer. In the reticular and hypodermic regions, arteriovenous anastomoses are common and play a role in thermoregulation of the body (Kierzenbaum 2002, Fawcett 2002, Young 2000).

![Fig. 3. The blood supply of the skin. Arteriovenous anastomoses are common in the cutaneous plexus and in the subcutaneous arteries and veins.](image-url)
Already in 1969, a cold toe was identified as a new and easily accessible parameter of the severity of circulatory shock (Joly and Weil 1969). Unlike in other forms of shock, in septic shock, the central to toe difference and microcirculatory alterations do not correlate (Boerma et al. 2008). This finding strengthens the theory regarding a dispersive nature of blood flow under conditions of sepsis between the microcirculatory and systemic hemodynamics.

Various studies have replicated the role of microcirculatory function in the pathophysiology of multi organ dysfunction (MODS) and sepsis. Despite the fact that discordance between systemic hemodynamic parameters and the microcirculatory alterations is most prominent during sepsis compared to other forms of shock, there is a wide heterogeneity in the results (De Backer et al. 2002). Microcirculatory alterations have been identified as markers for morbidity and mortality, whereas systemic hemodynamic parameters have failed to do so under septic conditions (De Backer et al. 2006, Sakr et al. 2004).

Although impaired capillary perfusion and the failure of improvement in microvascular dysfunction have been identified as risk factors of an adverse outcome in septic patients, the regional parameters of microvascular function do not reflect the severity of established MODS in hemodynamically stable patients (De Backer et al. 2006, Hans Knotzer et al. 2006, Sakr et al. 2004).

Reactive hyperemia response in the skin is diminished in patients with cardiogenic or septic shock, as well as in surgical intensive care patients (Haisjackl et al. 1990, Hartl et al. 1988, Kirschenbaum et al. 2000). In one study, the maximal vasodilator response to acetylcholine and sodium nitroprusside in the skin of patients suffering from septic shock was severely depressed when compared with nonseptic patients and healthy controls (Kubli et al. 2003).

2.5 The effect of systemic inflammatory response syndrome on wound healing

2.5.1 Definitions of SIRS, sepsis, severe sepsis, MODS and MOF

Systemic inflammatory response (SIRS) describes the systemic inflammatory process independent of its cause. The symptoms of SIRS are represented in Table 2 and a diagnosis includes at least more than one of the symptoms. The diagnosis presumes that these physiologic changes represent an acute alteration from the baseline in the absence of other known causes for similar abnormalities. These
terms were established in 1992 as part of the American College of Chest Physicians and Society of Critical Care Medicine Consensus Conference (Bone et al. 1992, Levy et al. 2003a, 2003b). In a recent multi-centre observational study the need for two or more SIRS criteria for a severe sepsis definition failed to identify one in eight patients from patients who were not fulfilling the SIRS criteria. The majority (74%) did not have an abnormal leukocyte count and 39% were postoperative (Kaukonen et al. 2015).

The terms of sepsis, severe sepsis and septic shock are defined according to the American College of Chest Physicians–Society of Critical Care Medicine consensus definition and are described in Table 2 (Levy et al. 2003b). Organ dysfunctions are defined using the Sequential Organ Failure Assessment (SOFA) score described in Table 3. The definitions of multiple organ dysfunction syndrome (MODS) and multi-organ failure (MOF) are also described in Table 2 (Vincent et al. 1996).

**Table 2. Definitions of SIRS, sepsis, severe sepsis, and septic shock. Bpm beats per minute; PaCO2 arterial partial pressure of carbon dioxide, BP blood pressure; temp temperature.**

<table>
<thead>
<tr>
<th>Term</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRS</td>
<td>temp &gt; 38°C or &lt; 36°C&lt;br&gt; tachycardia &gt; 90 bpm&lt;br&gt; respiratory rate &gt; 20 breaths/minute or PaCO2 &lt; 4.3 kPa&lt;br&gt; white blood count &gt; 12 000 mm$^3$ or &lt; 4 000 mm$^3$ or &lt; 10% immature forms</td>
</tr>
<tr>
<td>Sepsis</td>
<td>SIRS due to infections</td>
</tr>
<tr>
<td>Severe sepsis</td>
<td>Sepsis with evidence of organ hypoperfusion (organ dysfunction)</td>
</tr>
<tr>
<td>Septic shock</td>
<td>Severe sepsis with hypotension (Systolic BP &lt; 90 mmHg) despite adequate fluid resuscitation or the requirement for vasopressors/inotropes to maintain blood pressure</td>
</tr>
<tr>
<td>Organ dysfunction</td>
<td>SOFA score 1–2 in one organ system</td>
</tr>
<tr>
<td>Organ failure</td>
<td>SOFA score 3–4 in one organ system</td>
</tr>
<tr>
<td>MODS</td>
<td>SOFA score 1–2 in two or more organ systems</td>
</tr>
<tr>
<td>MOF</td>
<td>SOFA score 3–4 in two or more organ systems</td>
</tr>
</tbody>
</table>
Table 3. SOFA score. SOFA includes subscores’ ranging from 0 to 4 for each of six components, with higher scores indicating more severe organ impairment. Adrenergic agents are administered at least for 1 h and doses are given in microg/kg/min.

<table>
<thead>
<tr>
<th>Organ system</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration</td>
<td>PaO2/FIO2 &lt; 400</td>
<td>&lt; 300</td>
<td>&lt; 200 with respiratory support</td>
<td>&lt; 100 with respiratory support</td>
</tr>
<tr>
<td>Cardiovascular Hypotension</td>
<td>MAP &lt; 70 mmHg dopamine &lt; 5 or dobutamine in any dose</td>
<td>dopamine &gt; 5 or epinephrine &lt; 0.1 or norepinephrine &lt; 0.1</td>
<td>dopamine &gt; 15 or epinephrine &gt; 0.1 or norepinephrine &gt; 0.1</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.2–1.9</td>
<td>2.0–5.9</td>
<td>6.0–11.9</td>
<td>&gt; 12</td>
</tr>
<tr>
<td>Renal</td>
<td>1.2–1.9</td>
<td>2.0–3.4</td>
<td>3.5–4.9 or &lt; 500 ml/24 h</td>
<td>5.0 or &lt; 200 ml/24 h</td>
</tr>
<tr>
<td>Coagulation</td>
<td>Platelets x1000/mm³</td>
<td>&lt; 150</td>
<td>&lt; 100</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>CNS</td>
<td>Glasgow Coma Scale</td>
<td>13–14</td>
<td>10–12</td>
<td>6–9</td>
</tr>
</tbody>
</table>

2.5.2 Wound healing in severe sepsis

Sepsis

Septic patients are at an increased risk for problems related to poor wound healing, such as anastomotic leaks, fascial dehiscence, and nonhealing wounds (Rico et al. 2002). These complications may result in re-operations, prolonged intensive care, increased morbidity, and mortality, raising both the duration and the cost of care (Balk 2000, De Haan et al. 1974). Despite this well-known clinical problem, the molecular mechanisms that exist beneath the problem are still unknown (Rico et al. 2002, Sommer et al. 2013).

Animal models

In animal models, sepsis causes several alterations in wound healing. In experimental animals, both a systemic transient bacteraemia and a distant inflammation from intradermal croton oil result in impaired wound healing, and wound breaking strength and collagen content have been shown to be diminished.
(De Haan et al. 1974, Greenhalgh & Gamelli 1987, Houck & Jacob 1961, Stamm et al. 2000). Also, the neutrophilic recruitment to a remote site of secondary injury (skin) was shown to be reduced in septic mice (Swartz et al. 2000). In addition to a significant decrease in neutrophilic content (5% of control), a 30% reduction in the macrophage content was observed in the remote wounds of septic mice (Rico et al. 2002).

Sommer et al. showed that systemic sepsis causes a delay in mice wound healing in a remote wound without contact with the source of sepsis (Sommer et al. 2013). This phenomena has previously been shown in the work of other groups (Greenhalgh & Gamelli 1987, Marti et al. 2008, Rico et al. 2002). Rico et al. also observed a delayed re-epithelisation in septic mice in addition to a decreased wound collagen content (Rico et al. 2002).

Maish et al. found that inhibition of TNF by a TNF binding protein improves the altered wound healing process in colonic anastomosis impaired by sepsis, thus encouraging the hypothesis that TNF is involved in the delay of wound healing (Maish et al. 1998). In the same study, collagen deposition and organization decreased in cutaneous wounds during the septic state (Maish et al. 1998). Granulation tissue penetration and quality were decreased in septic rats, and the administration of specific TNF antagonist significantly attenuated the effects of sepsis on granulation tissue histology, but not to the control levels (Cooney et al. 1997). Recently, a decrease in local TNF and TGFβ expression in the wounds of septic mice was observed while the systemic serum levels of pro-inflammatory cytokines TNF and IL-6 were elevated (Sommer et al. 2013).

In a rat model comparing rapidly healing intestinal wounds to slower healing fascial and skin wounds, it was found that a consistent decrease in TNF was seen in healing intestinal tissues when compared to skin and fascia, but no clear pattern of increased anti-inflammatory or regulatory cytokines was seen (Zubaidi et al. 2010). In another rat model of an ischemic chronic wound with delayed healing, a sustained elevation of TNF levels was seen (Chen et al. 1999, Zubaidi et al. 2010).

Wounds in septic animals initially exhibited a decrease in matrix metalloproteinase 7 (MMP7) as well as a tissue inhibitor of matrix metalloproteinase 1 (TIMP1) expression, but during the later stages of wound healing, the TIMP1 levels increased although MMP levels equalled that of the control animals, thus suggesting that the deregulation of proteinases in wound healing during sepsis might be one mechanism that takes part in the inadequate inflammatory response after wounding and leads to impaired healing (Sommer et al. 2013). Greenhalgh et al. proposed already in 1987 in a study on wound repair
during sepsis that short-term nutritional depletion resulting from an infection can be a major determinant for impaired wound healing (Greenhalgh & Gamelli 1987). Nevertheless, in a later study, septic mice showed a delay in wound repair, whereas the non-septic pair-fed counterparts displayed normal wound healing (Marti et al. 2008).

**Human studies**

Clinical studies on skin wound healing in sepsis are rare. In a study of patients’ undergoing major surgery, when an operation led to diminished wound healing and this reduction in wound healing capacity was more pronounced in patients; having postoperative septic complications (Jorgensen et al. 1996). Septic patients will deliver over 70% less neutrophils to secondary inflammatory sites compared to healthy controls; however, no studies on white blood cells in wound healing in sepsis exists (Ahmed et al. 1999).

Further, patients with septic complications following injury demonstrated a reduced hydroxyproline accumulation compared to injured patients without septic complications (Clark et al. 2000). Diminished hydroxyproline accumulation was seen also in severe sepsis (Clark et al. 2000). In the same study, no effect of systemic TNF blockade on collagen accumulation was seen (Clark et al. 2000).

### 2.5.3 Systemic inflammation and wound healing in trauma and burns

For the restoration of tissue integrity, response to injury is an essential innate host immune response (Zaja-Milatovic & Richmond 2008). Wound healing proceeds via an overlapping pattern of events as described earlier, regardless of trauma, microbes, or foreign materials that initiate the process.

The burn injury disrupts the barrier function of the skin. It can induce a massive water loss that can rapidly cause deadly hypovolemic shock, depending on the size and depth of the burn injury (Blais et al. 2013). The burn wound exudate represents the burn tissue microenvironment and can be used in studies in the same way as blister fluid is used (Widgerow et al. 2015). Burns are characterized by local, intense inflammatory response that can impede healing (Washburn et al. 2013). Thus, the intense inflammation seen in burn wounds that the anti-inflammatory cytokines have discussed are potent targets of drug therapies. For example, TNF is often referred to as a powerful regulator of the inflammatory response and many clinically approved inhibitors of TNF are commercially available (Clark 2006).
Even though increased levels of IL-6 follow burns, sepsis, and other inflammatory conditions neutralizing IL-6 appear to have a mixed therapeutic benefit in pre-clinical models (Gallucci et al. 2000). Due to the complex functions of IL-6 in the inflammatory microenvironment, the window for neutralizing it as therapeutically effective may be narrow (Washburn et al. 2013). Therefore, it is especially important to understand the whole cascade and the overlapping processes, including cytokines, chemokines, and lymphocytes etc.

Traumatic injury, such as burn, leads to global changes in systemic immune response. These may include suppressed immune function and increased susceptibility to infection (Branski et al. 2009). Burn trauma is also associated with remote organ injury, affecting the lung, kidney, and gut (Davis et al. 2012, Magnotti & Deitch 2005, Mosier et al. 2010, Stollwerck et al. 2011). In critically ill burn patients, immune suppression may facilitate the translocation of gut-derived bacteria and contribute to the development of sepsis because of the relationship between burns and remote organ injury (Deitch et al. 2006, Magnotti & Deitch 2005).

2.5.4 Skin as an end organ in multiple organ failure

Complications that involve impaired wound healing, such as anastomotic leaks, fascial dehiscence, and infections are common in septic patients (Barriere & Lowry 1995). They present a special problem since patients that suffer from sepsis often require surgical interventions that in turn lead to further complications caused by impaired wound healing due to sepsis (Sommer et al. 2013).

One of the severe complications in sepsis is disseminated intravascular coagulopathy (DIC), wherein reduced anticoagulant capacity and inhibited fibrinolysis are opposed to a massive activation of coagulation leading to abundant intravascular fibrin formation and microvascular thrombosis (Markiewski et al. 2008, Zeerleder et al. 2005). This uncontrolled thrombosis causes widespread ischemic organ damage up to organ necrosis and clinically impresses as widespread skin necrosis named as purpura fulminans and MODS adversely affecting the prognosis of the sepsis and the patient (Dhainaut et al. 2005). In purpura fulminans, soon after the vascular occlusion, the endothelial cells become oedematous resulting in capillary dilation with congestion of the vessels by erythrocytes, seen as erythema and oedema at the site of the skin injury. Petechiae develop when the capillary dilatation leads to the extravasation of blood elements into dermis. As the ischemia and haemorrhage into dermis progress, ecchymoses develop followed by
the development of haemorrhagic bullae in the subepidermal area (Betrosian et al. 2006). The end result is coagulative necrosis of the dermal and subcutaneous tissues with extensive dermal haemorrhage, manifesting as gangrenous necrosis. The stage of necrosis is associated with high morbidity and mortality (> 50%) (Betrosian et al. 2006).

The skin measures approximately 2 square metres and receives one-third of the body’s circulating blood volume via the capillaries. Because the skin covers such a large area and receives only capillary blood flow, many factors affect the ability of the skin and its supporting structures to remain intact. These factors include those that affect tissue perfusion, such as haemoglobin levels, interstitial and lymphatic flow, oxygen supply and demand, and the presence of endotoxins (Bryant 2010).

Septic patients are at risk for developing pressure ulcers (PU). Hypoperfusion due to microvascular dysfunction, increased oxygen demand, and vasoconstriction in co-operation with immobility and decreased nitrogen balance can lead to the emergence of PUs or skin failure. Malnutrition, sedation, low blood pressure, and anemia are also risk factors for acute skin failure, defined as an event in which the skin and its underlying tissue die due to hypoprefusion concurrent with a critical illness (Langemo & Brown 2006). This phenomenon was noted by Reger et al. in an analysis of support surface interface pressure, Lamblin et al. in a study of 10 sedated ICU patients, and Campbell et al. in a review article on the metabolic response to trauma and sepsis (Campbell 1998, Lamblin et al. 2006, Reger et al. 2007).

Compromised skin integrity may be closely associated with mortality. A non-experimental, retrospective analysis of PU data involving 74 patients showed that patients who develop full-thickness PUs had a 180-day mortality rate of 68.9%, and those deaths were unrelated to the PUs — i.e., the development of a full-thickness PU was a precursor, not a cause, of death. Mortality rates ranged from 33% within 30 days to 73.3% within 1 year of onset of skin failure in this intensive care population.

Many septic patients are mechanically ventilated and require sedation. Sedative medications also cause changes in microcirculation that may alter tissue perfusion (Lamblin et al. 2006). Septic patients also frequently develop symptoms of poor nutrition, such as loss of lean tissue and reduced body mass that can affect also tissue perfusion and sepsis patients are especially at risk for malnutrition due to an alteration in metabolism and absorption (Campbell 1998).
2.6 Suction blister model

The Suction blister model was developed by Kiistala in 1968 (Kiistala 1968). In this model, a prolonged vacuum induces the disruption of dermo-epidermal junction and forms a blister. It separates the epidermis from the dermis while the basal lamina remains intact. A specific suction blister device is used to create the blister wounds (Mucel Ky, Nummela, Finland) (Fig 4). The device contains five 8 mm-diameter bores, and it is connected to the vacuum pump, which creates negative pressure to the area. The warming of the skin accelerates the blister formation. A higher vacuum (60–70 kPa) was used in the beginning, and after 20 to 30 minutes, a lower one (40–50 kPa). After blister induction, the blister fluid was collected, and the blister roofs were removed. The model is rather noninvasive, painless, and leaves no scars.

Fig. 4. The suction blister device (A) and the fully developed suction blisters (B) after induction.

The Suction blister method can be used to collect the blister fluid. The collected blister fluid has been shown to represent the interstitial fluid (Kiistala 1968, Vermeer et al. 1979). Collagen synthesis, various enzymes, and cytokines can be measured from the blister fluid using different assays and methods (Gäddnäs et al. 2010, Gäddnäs et al. 2009, Koivukangas et al. 2005, Leivo et al. 2000). This model can be also used to study re-epithelization and skin barrier regeneration (Koivukangas et al. 1999, Koivukangas & Oikarinen 2003, Malminen et al. 2003). The skin barrier is ruptured during induction, leading to increased water evaporation from the wound. As the stratum corneum does not exist in the new
epidermis, it has no effect on the water evaporation at that time (Kallioinen et al. 1995, Lévy et al. 1995).

The suction blister model creates a standardized superficial wound that enables the study of in vivo re-epithelisation, in which healing occurs along the partially restored basement membrane. The separation of the epidermal and dermal layers occurs just below the basal cells above the lamina densa layer of the basement membrane. Type IV collagen remains completely in the blister floor, but laminin-332 can be found variably in the blister floor and in basal cells of the detached epidermis (Koivukangas & Oikarinen 2003).

### 2.7 Methods studying blood flow response in sepsis

A laser Doppler flow meter (Periflux PF1; Perimed KB, Stockholm, Sweden) was first used in 1975 to measure skin blood flow in humans (Stern 1975). Since then, it has been widely used in studies and is considered a reliable method to use to measure skin blood flow (Choi & Bennett 2003, Obeid et al. 1990, Oberg et al. 1984). The laser beam penetrates about 1 mm into the skin. The vasculature of the skin contains two plexuses, but the laser Doppler reaches only the papillary plexus, which is the superficial one just beneath the dermo-epidermal junction (Tyler et al. 2001). Detection is not influenced by the blood flow of the underlying skeletal muscle (Braverman 2000, Eun 1995).

Increased wound blood flow (change from normal skin blood flow) in the wound is caused by inflammation and is considered a reliable parameter for overall wound inflammation (Broughton et al. 2006a, Li et al. 2007, Svedman et al. 1991). After wounding, a short vasoconstrictive phase is followed by vasodilatation, which peaks after a few days of healing and then calms down toward final healing (Broughton et al. 2006a).

In this study, all five blisters were measured, and the mean was calculated and reported. Also, the value from the healthy looking skin was measured and reported. Measurements were expressed as perfusion units, which is arbitrary.

### 2.8 Methods for studying transepidermal water loss in sepsis

Barrier recovery and re-epithelisation can be followed non-invasively by measuring water evaporation from the wound. Epidermal proliferation takes place from the edge of the wound, and migration covers the detective area (Broughton et al. 2006a). The changes in the cytoskeleton and the cell-to-cell and cell-to-matrix...
connections enable the migration. The process is controlled via cytokines originating from the epidermal cells, platelets, fibroblasts, and wound leukocytes (Li et al. 2007, Velnar et al. 2009). Since the epidermal barrier is a tightly regulated gateway to a percutaneous passage, TEWL (transepidermal water loss) decreases when the epidermal barrier is restored (Visscher et al. 2001).

After blister induction (when there is no epidermis), the water evaporation is 15- to 20-fold higher than in the intact skin. During the healing process, evaporation decreases, enabling the non-invasive follow-up of epidermal healing (Koivukangas & Oikarinen 2003, Lévy et al. 1995, Nilsson 1977, Svedman et al. 1991). In this study the transepidermal water loss was measured by using a VapoMeter (Delfin Technologies Ltd, Kuopio, Finland). The VapoMeter forms a closed chamber on the skin, and the sensors of humidity and temperature register the increase in relative humidity. The system automatically calculates the evaporation rate and gives the amount of water loss in grams per square metre. The VapoMeter has been shown to be a reliable device to use to measure TEWL (de Jongh et al. 2006, Fluhr et al. 2006). Portable and the small format allows for measuring in inconvenient circumstances.

VapoMeter is shown to be a reliable way to measure a skin barrier function (Fluhr et al. 2006). It’s recommended ambient conditions are the ones used: temperature 20–25 Celsius and relative humidity 10–60%. The device manufacturer reports that VapoMeters accuracy in measuring the evaporation rate through a semipermeable membrane is ± 10% (TEWL > 5 g/m²h) or 0.5 g/m²h (TEWL < 5 g/m²h). It is tested against the gravimetric evaporation rate (mass change of water samples covered with vapour permeable membrane) (http://www.delfintech.com/products_vapometer.html). The coefficient variation, when measured from the skin, is typically less than 10%.

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3 Aims of the study

The aim of this study is to offer new information about skin in sepsis and especially in wound healing. The most important tasks were to demonstrate the clinically obvious dysfunction in wound healing and find the key interpretative factors behind these phenomena.

In more detail the following questions were of particular interest:

1. Is the barrier restoration diminished and inflammation in the wound altered in severe sepsis by systemic inflammation?
2. Is the expression of basement membrane components (laminin-332 and type IV collagen) altered during severe sepsis and after 3 months in intact skin?
3. Is the expression of tight junction proteins (claudin-1, claudin-4, ZO-1 and occludin) disturbed in severe sepsis in wound healing?
4. Is the cytokine profile of the skin different from the cytokine profile of the serum in severe sepsis? How does the cytokine profile of the skin and serum compare to healthy controls?
4 Patients and methods

This prospective observational case-control study was carried out in the tertiary level medical-surgical intensive care unit at the Oulu University Hospital. The study was divided into sub-studies (studies I-IV) corresponding to the list of original publications. The study was conducted in collaboration with the departments of Anaesthesiology, Division of Intensive Care Medicine, Surgery and Dermatology of Oulu University Hospital, and the Medical Research Center of Oulu. The Department of Pathology was also involved in Studies II and III. Study III was conducted in collaboration with the Department of Dermatology of the University of Turku and Turku University Hospital. Study IV was performed in association with the Institute of Biomedicine and Biocenter of Oulu University Hospital. The study protocol was approved by the Ethics committee of the Oulu University Hospital (Register Number 50/2005). In addition, approval for the study was obtained from the Operative Division of the Northern Ostrobothnia Hospital District. The immunohistochemistry was performed in the Department of Pathology for Studies II and III. The laboratory work was done in the research laboratory of the Institute of Biomedicine and Biocenter of Oulu for Study IV.

4.1 Patients and the study design

From 10 May 2005 to 15 December 2006, 1361 patients were admitted to the ICU and screened for eligibility for the study. Inclusion criteria were a diagnosis of severe sepsis according to the criteria of ACCP/SCCM (Bone et al. 1992). The exclusion criteria included age under 18 or age over 80 years, bleeding disorder, or surgery not related to sepsis, malignancy, chronic liver failure, chronic kidney failure, immunosuppression and cortisone treatment not related to sepsis. Also an early death or transport to another hospital led to dropping. 238 patients fulfilled the inclusion criteria but 172 had to be excluded. Finally, the first sampling was done on 44 patients in 48 hours from the diagnosis of severe sepsis. Written informed consent was obtained from the patient or a legal surrogate in all cases. The controls were age-matched volunteers from a nearby residents’ house and a pension club. The description of the sub-studies is found in Table 4. In Study I, 35 patients were included in the analysis because of technical problems during the physiological measurements. In Study II the skin biopsies were taken from 20 patients and 4 controls. In study III healing suction blisters were collected from 15
patients and 10 control participants. In study IV, all 44 patients and 10 controls were included in the analysis.

4.1.1 Clinical data

The clinical data were prospectively collected from the electronic patient data management system (Centricity Critical Care Clinisoft, GE Healthcare, Helsinki, Finland) and the hospital records. The information collected from study patients included age, sex, chronic diseases, type of ICU admission (medical or surgical), reason for admission, focus of infection and severity of underlying diseases on admission, as assessed by the Acute Physiology and Chronic Health Evaluation II (APACHE II, Table 5) (Knaus et al. 1985, Knaus et al. 1981). In addition, the evolution of organ dysfunction was assessed for 10 days with a daily SOFA score (Table 3) (Vincent et al. 1996, Vincent et al. 1998). A daily SOFA score of 3 to 4 in two or more organ systems on one or more days during the study period was defined as multi organ failure (MOF). Additionally, a daily SOFA score of 1 to 2 in two or more organ systems on one or more days was defined as multiple organ dysfunction syndrome (MODS). The 30-day mortality was also recorded. Characteristics of all the study patients are seen in Table 6. All patients were treated according to the normal ICU protocol, according to severe sepsis guidelines, including insulin infusion to sustain blood glucose at a normal level and hydrocortisone supplementation in septic shock refractory to vasopressor therapy (Dellinger et al. 2004). The hydrocortisone dose used was 200 microg per day. Only 6 patients had a positive blood culture: Staphylococcus epidermidis, bacteroides fragilis, clostridium perfringens, hemophilus influenzae, klebsiella pneumoniae, and streptococcus pneumoniae.
Table 4. Description of the sub-studies. TEWL transepidermal water loss, BF blood flow.

<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
<th>Controls</th>
<th>Study question</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study I</td>
<td>35</td>
<td>15</td>
<td>Is the barrier restoration diminished and the inflammation increased in a local wound in sepsis?</td>
</tr>
<tr>
<td>Study II</td>
<td>20</td>
<td>4</td>
<td>Is the basement membrane component expression altered in severe sepsis?</td>
</tr>
<tr>
<td>Study III</td>
<td>15</td>
<td>10</td>
<td>Is the tight junction distribution normal in severe sepsis even though the restoration of the epidermal barrier is altered?</td>
</tr>
<tr>
<td>Study IV</td>
<td>44</td>
<td>15</td>
<td>Is there a difference between the skin and the serum cytokine profile?</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study</th>
<th>Quantities measured</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study I</td>
<td>TEWL, BF</td>
<td>Physiological measurements</td>
</tr>
<tr>
<td>Study II</td>
<td>Laminin-332 and Type IV collagen</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Study III</td>
<td>Tight junction proteins</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Study IV</td>
<td>Cytokines and Growth factors</td>
<td>Multiplexing cytokine assay</td>
</tr>
</tbody>
</table>
Table 5. The APACHE II scoring system. APACHE II is computed based on 11 measurements and GCS, age points, and chronic health points. Modified from Knaus et al. 1981.

<table>
<thead>
<tr>
<th>Physiologic variable</th>
<th>High abnormal range</th>
<th>Low abnormal range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Temperature - rectal (°C)</td>
<td>&gt; 41</td>
<td>39–40.9</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>&gt; 160</td>
<td>130–159</td>
</tr>
<tr>
<td>Heart rate per minute (ventricular response)</td>
<td>&gt; 180</td>
<td>140–179</td>
</tr>
<tr>
<td>Respiratory rate per minute (non-ventilated or ventilated)</td>
<td>&gt; 50</td>
<td>35–49</td>
</tr>
<tr>
<td>Oxygenation (mmHg)</td>
<td>a. FiO2 &gt; 0.5 use A-aDO2</td>
<td>&gt; 500</td>
</tr>
<tr>
<td></td>
<td>b. FiO2 &lt; 0.5 use PaO2</td>
<td>PO2 &gt; 70</td>
</tr>
<tr>
<td>Arterial pH (preferred) OR</td>
<td>&gt; 7.7</td>
<td>7.6 to 7.69</td>
</tr>
<tr>
<td>Serum HCO3 (venous mEq/l, not preferred)</td>
<td>&gt; 52</td>
<td>41–51.9</td>
</tr>
<tr>
<td>Serum sodium (mEq/l)</td>
<td>&gt; 180</td>
<td>1160–179</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl) double point score for acute renal failure</td>
<td>&gt; 7</td>
<td>6–6.9</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>&gt; 60</td>
<td>50–59.9</td>
</tr>
<tr>
<td>White blood count (total 1000/mm³)</td>
<td>&gt; 40</td>
<td>20–39.9</td>
</tr>
</tbody>
</table>

Glasgow Coma Scale Score = 15-actual GCS
A. Total Acute Physiologic Score (Sum of 12 above points)
B. Age points (years) < 45 = 0, 45 to 54 = 2, 55 to 63 = 3, 65 to 75 = 4 and > 75 = 6
C. Chronic health points: If the patient has a history of severe system insufficiency of is immunocompromised assign points as follows:
   a) for non-operative or emergency postoperative patient, 5 points; b) for elective postoperative patient, 2 points
Table 6. Patient demographics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ALL</th>
<th>Study I</th>
<th>Study II</th>
<th>Study III</th>
<th>Study IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient N</td>
<td>44</td>
<td>35</td>
<td>20</td>
<td>15</td>
<td>44</td>
</tr>
<tr>
<td>Male</td>
<td>29 (66%)</td>
<td>22 (63%)</td>
<td>15 (75%)</td>
<td>10 (67%)</td>
<td>29 (66%)</td>
</tr>
<tr>
<td>Age, median</td>
<td>63 (24 to 80)</td>
<td>62 (24 to 80)</td>
<td>63 (36 to 80)</td>
<td>64 (24 to 76)</td>
<td>63 (24 to 80)</td>
</tr>
<tr>
<td>APACHE II on admission, median</td>
<td>26 (9 to 44)</td>
<td>25 (9 to 44)</td>
<td>24 (14 to 42)</td>
<td>23 (9 to 37)</td>
<td>26 (9 to 44)</td>
</tr>
<tr>
<td>MOF</td>
<td>30 (68%)</td>
<td>23 (66%)</td>
<td>12 (60%)</td>
<td>9 (60%)</td>
<td>30 (68%)</td>
</tr>
<tr>
<td>Maximum SOFA score, median</td>
<td>10 (1 to 22)</td>
<td>9 (1 to 22)</td>
<td>8.6 (3 to 16)</td>
<td>8 (2 to 18)</td>
<td>10 (1 to 22)</td>
</tr>
<tr>
<td>30- day mortality</td>
<td>11 (25%)</td>
<td>10 (29%)</td>
<td>5 (25%)</td>
<td>2 (13%)</td>
<td>11 (25%)</td>
</tr>
<tr>
<td>Length of the stay in ICU, median</td>
<td>7 (1 to 30)</td>
<td>8 (2 to 30)</td>
<td>6 (1 to 21)</td>
<td>8 (2 to 17)</td>
<td>7 (1 to 30)</td>
</tr>
<tr>
<td>Hydrocortisone therapy</td>
<td>32 (73%)</td>
<td>24 (69%)</td>
<td>9 (55%)</td>
<td>7 (46.7%)</td>
<td>32 (73%)</td>
</tr>
<tr>
<td>Temperature on admission, Celsius</td>
<td>37.2 (34 to 40.3)</td>
<td>37.1 (34 to 40.3)</td>
<td>37.2 (35.9 to 40.3)</td>
<td>37.2 (36.1 to 38.3)</td>
<td>37.2 (34 to 40.3)</td>
</tr>
<tr>
<td>Leukocytes on admission</td>
<td>11.5 (0.3 to 55.3)</td>
<td>10.9 (0.3 to 55.3)</td>
<td>12.2 (7.4 to 24.9)</td>
<td>10.8 (4.7 to 19.6)</td>
<td>11.5 (0.3 to 55.3)</td>
</tr>
</tbody>
</table>
4.1.2 Induction of the blister wound and study protocol

Within the 48 hours of a diagnosis of severe sepsis, the first set of suction blisters were induced on healthy looking abdominal skin. After induction, the blister fluid was collected, and the blister roofs were removed. The TEWL and blood flow were measured from the blisters and from the healthy looking skin. Afterwards, the blisters were covered with an air and water vapour permeable self-adhesive wound dressing (Mepore, Mölnlycke Healthcare Ab). The measurements of TEWL and blood flow were repeated on the 4th day of healing (Table 7). The second set of suction blisters were induced on the 4th day from the start of the study. The same protocol was used as on the first day, and measurements were repeated on the 8th day from the beginning of the study. The venous blood samples were collected at the beginning of the study.

Table 7. Course of the study. TEWL transepidermal water loss, BF blood flow.

<table>
<thead>
<tr>
<th>Study days</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suction blister induction</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Blood samples</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

Skin biopsies

The skin biopsies were taken with a small biopsy scalpel 4 mm diameter on the first and the eighth day of the study in the intensive care unit. The samples were immediately fixed in a 10% phosphate buffered formalin solution. Further 16 biopsies were taken on the first and 12 on the eighth day of the study (see Table 8). Also, 7 biopsies were taken after 3 months.

Also, 15 healing, non-infected suction blisters were excised with a small scalpel in the intensive care unit from the third to seventh day of healing (see Table 8). Half of the sample was frozen immediately, and the other half was fixed in a 10% phosphate buffered formalin solution.

All skin samples were taken under local anaesthesia (1% lidocain) and the wounds were closed with sutures then removed after seven days. There were no complications in the healing of the wounds.
The control group consisted of 15 age-matched volunteers (7 males and 8 females). One set of suction blisters was induced to be controls and a venous blood sample was collected. The same device and method was used for both the control and septic patients.

### Table 8. The samples for immunohistochemical staining.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study II</td>
<td>4 mm biopsy</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Study III</td>
<td>Excised healing suction blister wound</td>
<td></td>
<td>16</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Septic</td>
<td></td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

### 4.2 Immunohistochemical studies of skin samples

#### 4.2.1 Basement membrane components Laminin-5 and Type IV collagen

Skin biopsies of 4 mm diameter were first fixed in 10% phosphate buffered formalin solution and then embedded in paraffin. For immunohistochemical staining, 4 µm paraffin sections were cut out. The antibodies used in this study are seen in Table 9. The staining was done according to manufacturer instructions. This technique involves the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen, a biotinylated secondary antibody that reacts with the primary antibody, an enzyme-labelled streptavidin, and a substrate-chromogen (LabVision Corporation, a part from Thermo Fisher Scientific, http://www.labvision.com/, visited 14.10.2009).

#### 4.2.2 Examination of tight junction proteins occludins, ZO-1, claudin-1, and claudin-4

We used the frozen half of the excised healing suction blister for an examination of tight junction proteins. Frozen sections of suction blisters were cut to 4 µm on superfrost+ glass slides. The samples were air dried for one hour at room temperature. After washing with PBS/Tween, the sections were incubated with antibodies (see Table 9) for 30 min at room temperature. After another wash with PBS/Tween, a Dako Envision kit (K5007) and DAB (3,3’ diamino benzidine) were
used for detection according to manufacturer recommendations with an incubation period of 30 minutes. The slides were then counterstained with haematoxylin.

<table>
<thead>
<tr>
<th>Study</th>
<th>Antigen Type</th>
<th>Antigen Type</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Detection kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study II</td>
<td>Type IV-collagen mouse monoclonal</td>
<td>Clone CIV 22, DakoCytomation Denmark AS, Denmark</td>
<td>1:100</td>
<td>UltraVision Large Volume Detection System/LabVision, HRP, Thermo Fisher Scientific Inc. Fremont, CA 94538, USA</td>
<td></td>
</tr>
<tr>
<td>Study II</td>
<td>Laminin-332 rabbit polyclonal</td>
<td>DakoCytomation</td>
<td>1:400</td>
<td>Ultra Vision Large Volume Detection System/LabVision</td>
<td></td>
</tr>
<tr>
<td>Study III</td>
<td>ZO-1 rabbit polyclonal</td>
<td>Zymed Laboratories, San Francisco, CA, USA</td>
<td>1:100</td>
<td>Envision/Dako, Dako Denmark A/S, Glostrup, Denmark and DAB (3,3' diamino benzidine)</td>
<td></td>
</tr>
<tr>
<td>Study III</td>
<td>occludin rabbit polyclonal</td>
<td>Zymed Laboratories</td>
<td>1:100</td>
<td>Envision/Dako and DAB</td>
<td></td>
</tr>
<tr>
<td>Study III</td>
<td>claudin-4 mouse monoclonal</td>
<td>Invitrogen, Zymed Life Technologies Ltd, UK</td>
<td>1:200</td>
<td>Envision/Dako and DAB</td>
<td></td>
</tr>
<tr>
<td>Study III</td>
<td>Claudin-1 rabbit polyclonal</td>
<td>Invitrogen</td>
<td>1:200</td>
<td>Envision/Dako and DAB</td>
<td></td>
</tr>
</tbody>
</table>

4.3 Cytokine assay of the suction blister fluid

The cytokine levels of serum and blister fluid samples were analysed using a Bio-Plex 200 System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA) and a Milliplex Human Cytokine/Chemokine Magnetic Bead Panel (Cat# HCYTOMAG-60K-07, Millipore Corporation, Billerica, MA, USA). The levels were analysed using multiplexing, and measurement was performed according to manufacturer instructions (Johansson-Persson et al. 2014, Lehto et al. 2010, Myhrstad et al. 2011). The assay conditions were pre-standardized and optimized to ensure optimal reproducibility. The results were automatically calculated using Bio-Plex Manager Software 6.0 with five parameter logistic equations. The concentrations of IL-4, IL-6, IL-10, TNF, bFGF, VEGF, and EGF were analysed from the suction blister fluid obtained on the first day of study as well as the serum samples from the septic patients and the controls.
4.4 Statistical Methods

SPSS (version 15.0 to 22, SPSS Inc., Chigaco, IL, USA) was used for statistical analysis.

The data were entered into an SPSS database (SPSS Data Entry, Version 3.0; SPSS Inc., Chicago, IL, USA). Summary statistics are expressed as a median with the 25th and 75th percentiles or mean with a standard deviation (SD) and range. The analysis between the groups was done using the Kruskal-Wallis test. The Mann-Whitney U test was applied to analyse the differences between the two study groups. The categorical variables were analysed by the Fischer exact test. Spearman correlation was calculated. Two-tailed $P$ values were reported, and the analyses were performed using SPSS software (Version 18.0; SPSS Inc.). The differences were considered significant at $P$ values of less than 0.05. The medians and the $P$ values were reported.
5 Results

5.1 Blister wound healing in sepsis (I)

5.1.1 Restoration of the epidermal barrier function (I)

Based on the findings of this study, the decrease of the transepidermal water loss (TEWL) during healing reflects the restoration of the epidermal barrier function. The TEWL with standard deviations are reported in Table 10. The decrease of transepidermal water loss from day 0 to day 4 in the early wound was lower in the septic group than in the control group (56 vs. 124 g/m²/h, respectively). The same trend was also seen in the late wound (77 vs. 124 g/m²/h, p = 0.091).

There were no differences in the transepidermal water loss of the intact skin at any point in time between the septic patients and the control subjects. Neither the wound nor the intact skin showed any significant difference in transepidermal water loss between those patients who received or those who did not receive steroid treatment due to sepsis.

Table 10. Transepidermal water loss and blood flow in the early and in the late wounds.
SD in parenthesis, TEWL values are expressed as grams per square meter per hour and BF is expressed in perfusion units.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day</th>
<th>Transepidermal water loss</th>
<th>Blood Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Septic</td>
</tr>
<tr>
<td>Intact skin</td>
<td>Day 0</td>
<td>14 (17)</td>
<td>10 (7)</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>14 (17)</td>
<td>10 (7)</td>
</tr>
<tr>
<td>Early wound</td>
<td>Day 0</td>
<td>195 (30)</td>
<td>168 (58)</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>73 (31)</td>
<td>102 (93)</td>
</tr>
<tr>
<td></td>
<td>The decrease</td>
<td>124 (31)</td>
<td>56 (91)</td>
</tr>
<tr>
<td>Late wound</td>
<td>Day 0</td>
<td>195 (30)</td>
<td>180 (88)</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>73 (31)</td>
<td>86 (45)</td>
</tr>
<tr>
<td></td>
<td>The decrease</td>
<td>124 (31)</td>
<td>77 (63)</td>
</tr>
</tbody>
</table>

5.1.2 Blood flow response (I)

The blood flow (BF) from the suction blister wound and the intact skin is represented in Table 10. The mean BF on the fourth day of healing in the early wound was significantly higher in the septic group (47 vs. 110 units, p = 0.001, respectively). In the late wound, the mean BF after blister induction and on the
fourth day of healing was higher in septic patients than in the control subjects (51 vs. 101 units, \( p = 0.001 \), Fig 7, and 47 vs. 110 units, \( p = 0.005 \), respectively). On the fourth day, the mean blood flow from the intact skin was higher in the septic group (24 vs. 6 units, \( p < 0.001 \), respectively). The BF values did not differ between those patients who received and those who did not receive steroid treatment.

5.2 Skin biopsy (II)

In samples obtained from the intact skin of patients with severe sepsis, laminin-332 expression was weak or absent in 41% (7/17) on Day 1 and 45% (5/11) on Day 8 compared to 0% (0/4) of healthy control subjects (Table 11). At 3 months, the weak or absent laminin-332 expression was found in 14% (1/7) of the patients with severe sepsis.

Type IV collagen expression was weak or absent in 73% (11/15) of the intact skin samples obtained on Day 1 and 91% (10/11) on Day 8, compared to 0% (0/4) of the healthy control subjects (Table 11). At 3 months, the proportion of patients with weak or absent expression was 57% (4/7).

| Table 11. The intensity of laminin-332 and Type IV collagen staining in intact skin, assessed as a proportion of patients. NA, Not applicable; D1, Day 1; D8, Day 8; 3Mo, and 3 months. |
| Variable                  | Septic patients | Controls |
| Number of Patients       | Absent/Moderate | Moderate/Strong | Number of Patients | Absent/Moderate | Moderate/Strong |
| Laminin-332 D1           | 17              | 4 (41%)         | 10 (59%)          | 4              | 0 (0%)          | 4 (100%)        |
| Laminin-332 D8           | 11              | 5 (45%)         | 6 (55%)          | NA             | NA              |
| Laminin-332 3Mo          | 7               | 1 (14%)         | 6 (86%)          | NA             | NA              |
| Collagen IV D1           | 15              | 11 (73%)        | 4 (27%)          | 4              | 0 (0%)          | 4 (100%)        |
| Collagen IV D8           | 11              | 10 (91%)        | 1 (9%)           | NA             | NA              |
| Collagen IV 3Mo          | 7               | 4 (57%)         | 3 (43%)          | NA             | NA              |

5.2.1 Association with mortality (II)

Patients with weak or absent laminin-332 expression on study admission had a 30-day mortality of 43% (3/7) compared to 10% (1/10, \( p = 0.250 \)) in patients with moderate or strong expression with a comparable mean APACHE II score on admission (26.6 vs. 25.8, \( p = 0.835 \)). Patients with weak or absent Type IV collagen expression had a 30-day mortality of 33% (3/9) in comparison to 0 deaths (0%, 0/4,
p = 0.510) in patients with moderate or strong expression and a comparable APACHE II score on admission (23.1 vs. 27.8, p = 0.225).

5.3 Expression of cytokines and growth factors in sepsis (IV)

5.3.1 Cytokines and growth factors in blister fluid

In blister fluid IL-10, TNF and IL-6 levels were higher in severe sepsis compared to the controls (65.94 vs. 4.25 picog/ml, p < 0.001, 16.86 vs. 5.93 picog/ml, p = 0.045 and 41.93 vs. 0.03 picog/ml, p < 0.001, respectively) (Table 12). The levels of IL-10 in blister fluid correlated to the IL-10 levels measured in serum (Spearman’s rho 0.602, p < 0.001, respectively) as well as IL-6 and IL-4 levels (Spearman’s rho 0.550, p = 0.001 and Spearman’s rho 0.452, p = 0.04) (Table 13). There was no correlation between the blister fluid levels and the serum levels of EGF, VEGF, TNF, and bFGF (Table 13).

5.3.2 Systemic cytokine and growth factor response in sepsis

The IL-10, IL-6 and TNF levels in serum in severe sepsis were higher compared to the controls (25.72 vs. 4.45 picog/ml, p = 0.004, 45.52 vs. 2.10 picog/ml, p < 0.001 and 5.83 vs. 0.67 picog/ml, p < 0.001, respectively) (Table 12). In serum EGF levels were lower in the septic group compared to the controls (1.39 vs. 97.61 picog/ml, p < 0.001).
Table 12. Serum and blister fluid cytokine levels in septic patients and in controls. Serum and blister fluid cytokine levels expressed as medians (pg/ml) with the interquartile range in parentheses.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Serum</th>
<th>Blister fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Septic patients</td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>Median (interquartile range)</td>
<td>Median (interquartile range)</td>
</tr>
<tr>
<td>EGF</td>
<td>1.39 (1.39–1.39)</td>
<td>97.61 (28.23–177.96)</td>
</tr>
<tr>
<td>FGF2</td>
<td>20.93 (1.76–43.25)</td>
<td>22.74 (10.28–33.02)</td>
</tr>
<tr>
<td>IL-10</td>
<td>25.72 (9.15–132.71)</td>
<td>4.45 (2.12–18.04)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.07 (0.07–3.46)</td>
<td>2.18 (0.07–22.08)</td>
</tr>
<tr>
<td>TNF</td>
<td>5.83 (2.56–13.09)</td>
<td>0.67 (0.57–0.82)</td>
</tr>
<tr>
<td>VEGF</td>
<td>113.87 (14.74–237.24)</td>
<td>54.71 (20.87–60.36)</td>
</tr>
<tr>
<td>IL-6</td>
<td>45.52 (23.39–203.09)</td>
<td>2.10 (9.3–501.93)</td>
</tr>
</tbody>
</table>

Table 13. Correlation of the blister fluid cytokine levels with the serum cytokine levels. The levels of cytokines in blister fluid and in serum are reported as medians with interquartile range in parentheses. Spearman’s correlation co-efficiency and p-values are also reported. Asterisks represent statistically significant findings (p < 0.05).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Blister fluid</th>
<th>Serum</th>
<th>Spearman’s rho</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>1.39 (1.39–1.39)</td>
<td>1.39 (1.39–1.39)</td>
<td>0.37</td>
<td>0.019*</td>
</tr>
<tr>
<td>bFGF</td>
<td>21.32 (1.76–34.70)</td>
<td>20.93 (1.76–43.25)</td>
<td>0.276</td>
<td>0.089</td>
</tr>
<tr>
<td>IL-10</td>
<td>65.94 (14.52–210.54)</td>
<td>25.72 (9.15–132.71)</td>
<td>0.602</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.07 (0.07–2.04)</td>
<td>0.07 (0.07–3.46)</td>
<td>0.452</td>
<td>0.004*</td>
</tr>
<tr>
<td>TNF</td>
<td>16.86 (10.58–28.11)</td>
<td>5.83 (2.56–13.09)</td>
<td>0.202</td>
<td>0.218</td>
</tr>
<tr>
<td>VEGF</td>
<td>113.87 (14.74–237.24)</td>
<td>113.87 (14.74–237.24)</td>
<td>-0.099</td>
<td>0.547</td>
</tr>
<tr>
<td>IL-6</td>
<td>45.52 (23.39–203.09)</td>
<td>45.52 (23.39–203.09)</td>
<td>0.55</td>
<td>0.001*</td>
</tr>
</tbody>
</table>
Table 14. Serum and blister fluid cytokine levels in patients with MOF and patients without MOF (non-MOF). Median is reported with the interquartile range in parentheses. Asterisks represent the significant findings.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Serum</th>
<th>Blister fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOF</td>
<td>non-MOF</td>
</tr>
<tr>
<td>EGF</td>
<td>1.39</td>
<td>97.6</td>
</tr>
<tr>
<td></td>
<td>(1.39–7.81)</td>
<td>(28.2–178.0)</td>
</tr>
<tr>
<td>bFGF</td>
<td>20.93</td>
<td>21.99</td>
</tr>
<tr>
<td></td>
<td>(6.50–46.68)</td>
<td>(1.76–39.74)</td>
</tr>
<tr>
<td>IL-10</td>
<td>36.60</td>
<td>8.94</td>
</tr>
<tr>
<td></td>
<td>(17.06–196.68)</td>
<td>(6.00–26.89)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(0.07–2.89)</td>
<td>(0.07–4.42)</td>
</tr>
<tr>
<td>TNF</td>
<td>5.96</td>
<td>5.76</td>
</tr>
<tr>
<td>VEGF</td>
<td>90.85</td>
<td>141.85</td>
</tr>
<tr>
<td>IL-6</td>
<td>48.48</td>
<td>34.76</td>
</tr>
<tr>
<td></td>
<td>(27.95–562.23)</td>
<td>(17.01–84.62)</td>
</tr>
</tbody>
</table>

Table 15. Serum and blister fluid cytokine levels in survivors and non-survivors. Median reported with the interquartile range in parentheses. Asterisks represent the significant findings.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Serum</th>
<th>Blister fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nonsurvivors</td>
<td>survivors</td>
</tr>
<tr>
<td>EGF</td>
<td>1.39</td>
<td>1.39</td>
</tr>
<tr>
<td>bFGF</td>
<td>18.79</td>
<td>21.99</td>
</tr>
<tr>
<td></td>
<td>(1.76–48.18)</td>
<td>(2.20–43.07)</td>
</tr>
<tr>
<td>IL-10</td>
<td>88.49</td>
<td>19.32</td>
</tr>
<tr>
<td></td>
<td>(25.69–286.51)</td>
<td>(8.45–50.51)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(0.07–1.58)</td>
<td>(0.07–3.67)</td>
</tr>
<tr>
<td>TNF</td>
<td>9.56</td>
<td>5.76</td>
</tr>
<tr>
<td></td>
<td>(2.31–19.36)</td>
<td>(2.86–12.28)</td>
</tr>
<tr>
<td>VEGF</td>
<td>14.74</td>
<td>152.17</td>
</tr>
<tr>
<td></td>
<td>(1.63–61.64)</td>
<td>(74.00–257.13)</td>
</tr>
<tr>
<td>IL-6</td>
<td>91.13</td>
<td>38.53</td>
</tr>
<tr>
<td></td>
<td>(76.16– (15.73–68.04)</td>
<td></td>
</tr>
</tbody>
</table>
5.3.3 MOF vs. non-MOF

Thirty patients (68%) developed multi-organ failure (MOF). IL-10 levels in the blister fluid were higher among those with organ failures than for those without (116.36 vs. 21.27 picog/ml, p = 0.015) (Table 14). Also, in blister fluid, IL-4, IL-6 and bFGF levels were higher in patients with MOF (0.7 vs. 0.07 picog/ml, p = 0.013, 93.62 vs. 13.93 picog/ml, p = 0.039 and 25.92 vs. 9.47 picog/ml, p = 0.027) (Table 14). IL-10 levels in the serum were higher in patients with organ failures (36.60 vs. 8.94 picog/ml, p = 0.005, respectively).

5.3.4 Survivors vs. non-survivors

Total 30-day mortality was 25% (11 patients). The levels of cytokines in blister fluid and serum in survivors and non-survivors are seen in Table 15. Survivors had lower IL-10 and bFGF levels in blister fluid than did patients who did not survive (43.27 vs. 181.91 picog/ml, p = 0.024 and 15.84 vs. 31.85 picog/ml, p = 0.006) (Table 15). In serum, survivors had higher levels of VEGF and lower IL-6 levels than did non-survivors (152.16 vs. 14.74 picog/ml, p = 0.012 and 38.52 vs. 91.13 picog/ml, p = 0.011) (Table 15).

5.4 Tight junctions (III)

5.4.1 Intact skin

The healthy looking skin on the edge of the sample represents intact skin in this study. From the 3rd day of healing onward, occludin expression was strong and clear in the stratum granulosum, and it remained strong until the 7th day in the controls (Fig 5). The same effect was also seen with ZO-1 and claudin-4. No specific difference was found between the controls and the septic patients. Claudin-1 expression was strong in all epidermal layers in the controls and also in the samples from the septic patients (see Fig 6).

5.4.2 The hyperproliferative zone

At the border of the blister in the hyperproliferative zone occludin expression was strong and diffuse compared to the intact skin, and staining was also strong in the intercellular space. The expression was similar from the 3rd to the 7th day of healing,
and no difference was found between septic and controls. The expression of claudin-1 in septic patients was similar for all time points on the hyperproliferative zone, and the intercellular space was more intense compared to the intact skin (Fig 6). The expression was similar compared to the controls. The ZO-1 expression was more diffuse and seen in all layers of epidermis in the hyperproliferative zone compared to the intact skin (Fig 7). The phenomenon was milder for the septic patients compared to the controls. Claudin-4 expression in the hyperproliferative zone was more diffuse compared to the intact skin and was seen mainly in the uppermost layers of epidermis in the intercellular space.

Fig. 5. Occludin expression on the 7th day of healing in a septic patient (A) and in a control person (B). In sepsis, occludin expression was seen mainly in SG (A) whereas in the controls broadened expression was seen in multiple layers of epidermis.

Fig. 6. Claudin-1 expression on the 7th day of healing in a septic patient. Claudin-1 expression was strong in all the epidermal layers for all time points on the leading edge (B) and in the whole wound (A).
Fig. 7. The expression of ZO-1 on the 7th day of healing in a control (A and B) and in a septic patient (C).
5.4.3 The leading edge of the migrating keratinocytes

On the leading edge of migrating keratinocytes, claudin-1 expression was similar compared to the hyperproliferative zone. Claudin-1 was expressed in all epidermal layers on the leading edge of the samples in both the controls and the septic patients (Fig 9). ZO-1 was expressed in the intercellular space also nearly in all epidermal layers on the leading edge of the migrating keratinocytes (Fig 10). Claudin-4 was also expressed in intercellular space in several upper layers of epidermis in both the control group and the septic patients. Claudin-1, ZO-1 and claudin-4 expression was similar from the 3rd to the 7th day of healing. Occludin expression on the leading edge of the migrating keratinocytes was seen mainly in stratum granulosum (SG) in the septic patients (Fig 8). The same effect was seen from the 3rd to 7th day of healing. In the controls the occludin expression was similar on the 4th day of healing, but on the 7th day, when the wound was nearly healed, the expression was seen in several cell layers on the leading edge of the migrating keratinocytes (Fig 8). Also, on the 7th day of healing in the controls the occludin was expressed in the very first point of the leading edge, whereas that expression was not obvious on the 4th day of healing or with the septic patients.
6 Discussion

6.1 Main results and generalizability of the study

In this study, epidermal healing was disturbed, local inflammation was more intense, and the expression of cytokines was altered in skin in severe sepsis compared to skin for the healthy controls. Also, the basement membrane component expression was diminished in intact skin, while the tight junction proteins were well expressed in the healing wound in severely septic patients. Together, all these findings suggest that skin dysfunction in severe sepsis exists even though the most profound structures remain intact.

These are novel findings. Therefore, there are no previous human studies concerning wound healing or tight junctions in severe sepsis. Our findings offer a basic view to the local reactions of the skin to a systemic process, namely, sepsis.

The study group well reflected the typical medical-surgical intensive care unit severely septic patient even though some limitations existed. The patients were severely ill according to the SOFA and APACHE scores, and they had typical comorbidities. The majority were elderly people. Our study group was very similar compared to many other severe sepsis studies, but to minimize the effect of some chronic diseases, we had to exclude some patients. Therefore, these results cannot be generalized to touch those that suffer conditions listed in the exclusion criteria. The wounds studied were experimental non-infected wounds.

The study group was limited, however, and a larger study group would have definitely increased the value of the results. The study setting itself in the ICU with the suction blisters was demanding, and so collecting a larger amount of material would have been taken too long. Performing a multi-centre study with this kind of study setting is nearly inconceivable unless you have other centres familiar with the method being used.

6.2 Discussion of main results

6.2.1 Epidermal barrier restoration in blister wound model in severe sepsis

The restoration of the epidermal barrier was delayed in patients with severe sepsis compared to the control subjects. This phenomenon was seen in the early wound
(first to fourth day of the disease) as well as in the late wound (fourth to eighth day of the disease). There were no differences in the TEWL of the intact skin.

We used a closed chamber system to measure the transepidermal water loss. Compared to the open chamber systems, it has some advantages. It extends the measurement range to high evaporation rates despite the anatomical site or angle. Also the effect of external or body-induced airflows can be avoided (Nuu tinen 2003).

There are several aspects of severe sepsis that can modulate the restoration of the epidermal barrier function. Epidermal proliferation begins at the edge of the wound and at the dermal appendages. The process is dependent on cytokines, the growth factor, and other mediators originating from the epidermal cells, platelets, fibroblasts, and wound leukocytes (Jacinto et al. 2001, Martin 1997).

The observed delay in a restoration of the epidermal barrier in late severe sepsis could be related to a suppressed macrophage function and lack of growth factors. It has been shown that in late phases of septic disease some patients develop compensatory an anti-inflammatory response, characterised by a depressed immune system and defects in macrophage cytokine secretion (Döcke et al. 1997, Mokart et al. 2002, Song et al. 2008, Xiao et al. 2006). Since macrophages are the most important leucocytes in wound healing, it is possible that this defect impairs wound healing. However, in this current study, the delay was seen much earlier than when the compensatory anti-inflammatory response usually appears.

It is also possible that some increase in TEWL comes from increased capillary permeability during sepsis. However, the control patients had higher TEWL after blister induction than the septic patients did, which suggests that increased vascular permeability does not have a notable effect on TEWL.

6.2.2 Skin blood flow in the experimental wound and in intact skin

We found that the blood flow was higher in severely septic patients when compared to the controls on the fourth day of the early wound and on the first and on the fourth day of the late wound. This means that at the beginning of the disease, the blood flow is nearly similar but when the disease proceeds differences will appears.

Abdominal skin in our study represents the area with nutritive perfusion subserved by small capillaries. Nutritive areas are not as sensitive to body and room temperature changes as arteriovenous areas (Rendell et al. 1998). Also nutritive areas do not reflect systemic changes in blood circulation as easily as arteriovenous areas will (Rendell et al. 2000).
Inflammation is the first response to wounding. Inflammatory mediators recruit leukocytes to the wound area, which then cleanse the wound from microbes and damaged tissues (Broughton et al. 2006a). Impaired inflammatory response leads to a disturbed process of healing (Williams & Harding 2003). Inflammatory cell infiltrate is also diminished in wound healing in septic animals (Rico et al. 2002). Measurement of the level of local inflammation is not straightforward since the phenomenon itself is complex. Inflammation causes vasodilatation that results in increased blood flow; thus, the extent of the local inflammation can be estimated indirectly by measuring the level of blood flow (Choi & Bennett 2003). It is also possible that high systemic levels of inflammatory mediators increase local inflammation, supported by the fact that in our study BF was highest in the surgical patients, which were shown to have the most profound systemic inflammation (Lowry et al. 2004).

Several cytokines and growth factors have been associated to vascular response. In one prospective study, VEGF levels were increased in sepsis; and sepsis has been shown to be associated with a time dependent increase in circulating vascular growth factor (Shapiro et al. 2008, Yano 2006). Further, VEGF has been shown to have a role in increased microvascular permeability in septic patients and VEGF levels to positively correlate to the severity of organ dysfunction in severe sepsis (van der Flier et al. 2005, Pickkers et al. 2005).

Local effects of nitric oxide (NO) may also be related to increased wound blood flow. In sepsis, NO levels increase (Maxime et al. 2007). NO is a mediator of early wound healing and inflammation.

Our finding is supported by the previous study by Kubli et al. where sepsis patients did not differed from their controls in the first 48 hours (Kubli et al. 2003). Excess of the pro-inflammatory mediators, TNF, IL-1 and IL-6 have been demonstrated in sepsis, and correlated with prognosis (Kim & Deutschman 2000, Williams & Harding 2003). Also, one earlier study has shown that disturbed skin microcirculation in severe sepsis, and the level of disturbance correlated with MODS (H Knotzer et al. 2007). Late sepsis is also associated with variable inflammatory response: Some patients have sustained hyperinflammation and others, immunosuppression (Osuchowski et al. 2006). We further found an increased blood flow in late severe sepsis in the entire patient group, but then diminished blood flow in the most severely ill patients.

It has been shown that if organ failures resolve after two days of surgical treatment of abdominal infection, the patient has a favourable prognosis (Paugam-Burtz et al. 2002). The observed diminishing wound inflammation over time could
be related to the lack of mediators in the late phase of septic disease or tissue hypoxia. This explanation can only be hypothesized since these mediators were not measured in this study. There was no correlation between blood leukocyte levels and TEWL or BF in an early or late wound.

In the earlier studies, blood flow and TEWL have been shown to correlate positively in those patients with no systemic inflammatory disease. When the epidermal barrier is restored, the inflammation has also been shown to subside (Koivukangas et al. 1999). In this current study with severely septic patients, no such correlation was found which could be related to systemic inflammation in our patients.

6.2.3 Basement membrane component expression in severe sepsis

Type IV collagen acts as a critical microenvironmental factor in the basement membrane, which is needed to sustain keratinocyte growth and survival and optimize epithelial architecture (Segal et al. 2008). If the basement membrane is injured, then laminin-332 production increases rapidly. It then serves as a scaffold for cell migration, initiates the formation of hemidesmosomes, and accelerates basement membrane restoration at the dermal-epidermal junction (Schneider et al. 2007).

After an injury, adjacent keratinocytes are exposed to dermal collagens. Within a few hours, these cells secrete large amounts of laminin-332, probably as a response to activation by inflammatory cytokines, which is then followed by the disassembly of cell junctions (Decline et al. 2003, Nguyen et al. 2000). The laminin-332 deposited in the fresh wound was found to be unprocessed, whereas in the healthy skin, only processed laminin-332 could be detected (Nguyen et al. 2000a). Keratinocyte migration on laminin-332 depends on a variety of factors that control the remodelling of the actin cytoskeleton and redistribution of the integrins. Laminin-332 processing appears to play only a minor role for cell migration (Schneider et al. 2007).

Fibroblasts facilitate wound closure, but they differentially affected the deposition of various basement membrane components. The deposition of laminin-332 at the dermal-epidermal junction was delayed in superficial wounds when compared to the full-thickness wounds in human skin equivalents (El Ghalbzouri et al. 2004). In the same study laminin-332 and type IV collagen deposition was shown was as decreased in the laterally expanding epidermis, indicating that the
presence of these proteins is not required for keratinocyte migration to occur in vitro (El Ghalbzouri et al. 2004).

In our patients, laminin-332 expression was similar to the controls on the first day, but on the eighth day, expression decreased. In normal wound healing when the basement membrane is injured, laminin-332 production increases rapidly. Within a few hours, the keratinocytes, now exposed to dermal collagens, secrete large amounts of laminin-332, as a response to activation by the inflammatory cytokines (Déclère et al. 2003, Nguyen et al. 2000). Sepsis is characterized by a surge of the pro-inflammatory cytokines TNF and IL-1 in the early stage (Shimaoka & Park 2008). As the disease progresses, the hyper-inflammatory state during the early stage converts to the anti-inflammatory state, seen as decreased levels of TNF and increased levels of IL-10 (Rice & Bernard 2005, Russell 2006). The increased production of IL-10 in the late phase of sepsis is believed to contribute to the ‘immunosuppression’ (Scumia & Moldawer 2005). This effect could explain the decreased expression of laminin-332 and type IV collagen compared to the first day of septic disease.

Laminin-332 promotes epidermal attachment by increasing the rate of basement membrane formation, whereas a lack of laminin-332 can lead to diminished epidermal-dermal stability (Nishiyama et al. 2000). Human anti-laminin-332 autoantibodies have been shown to induce subepidermal blister formation on an experimental human skin graft model (Lazarova et al. 2000). The clinical findings correlated with the dose of anti-laminin-332. Severe sepsis itself clinically associates with skin fragility. In this study, in severe sepsis, laminin-332 expression was diminished during wound healing from the fourth day onward, compared to the controls and also was seen in the intact abdominal skin on the eighth day of study. It is further suggested that laminin-332 may improve epidermal attachment in a variety of healing processes or clinical situations where the epidermal–dermal attachment and basement membrane formation might be compromised (Nishiyama et al. 2000).

In this study, in the epidermal wounds, type IV collagen expression was more prevalent in severe sepsis compared to the controls. Type IV collagen is needed to sustain keratinocyte growth and survival and optimize epithelial architecture (Segal et al. 2008). Although the presence of laminin-5 and type IV collagen is not required for keratinocyte migration to occur in vitro, both are essential for the basement membrane formation (El Ghalbzouri et al. 2004). Type IV collagen is the major collagenous component of all basement membranes (Kashtan 1999). Degradation of Type IV collagen by MMP-2 plays a positive role in epithelial
healing by enabling the detachment and migration of epithelial cells; on the other hand, continuous dissolution of the basement membrane can prevent the epithelial repair (Gäddnäs et al. 2010).

We have previously shown that MMP-2 levels are elevated in sepsis, and the levels were higher in non-survivors (Gäddnäs et al. 2010). Elevated levels of MMP-2 are associated with septic organ damage in the skin, heart and lungs (Torii et al. 1997, Wohlschlaeger et al. 2005). Taken together, degradation of Type IV collagen may play a role that explains compromised basement membrane formation in severe sepsis.

6.2.4 Skin blister fluid cytokine expression in sepsis

Our human study showed a dysregulated wound inflammatory response, a novel finding since local cytokine expression in the skin has not been previously studied in severe sepsis. Previously, animal studies have shown that persistent elevation of pro-inflammatory cytokines is associated with delayed healing (Chen et al. 1999, Zubaidi et al. 2010).

The local IL-6 levels were higher in severe sepsis compared to the controls. Increased local skin blister levels of IL-6 have been previously associated with a number of inflammatory skin pathologies, such as psoriasis (Grossman et al. 1989, Werner & Grose 2003). The overexpression of IL-6 in the skin of normal rats induces epidermal proliferation and inflammation, while transgenic mice overexpressing IL-6 display a thickened stratum corneum (Luckett-Chastain & Gallucci 2009, Turksen et al. 1992).

In our series, IL-10 levels were higher in severe sepsis compared to the controls in blister fluid. In the murine model, it has been suggested that IL-10 may play an important regulatory role in the infiltration of neutrophils and macrophages as well as cytokine production in the inflammatory response of cutaneous wound healing (Sato et al. 1999).

In non-survivors and in patients with MOF, higher local levels of bFGF were obtained in blister fluid. This finding may mirror the adverse effects of cytokine stimulation in many organs – bFGF is known to be a potent stimulator of local inflammation and cell metabolism as well as growth of an extracellular matrix in the whole body. However, in sepsis, its effects may be misdirected (Beenken & Mohammadi 2009, Keller et al. 2008). Previously local bFGF overexpression in skin was described as being in patients with systemic sclerosis and one study on serum (Kadono et al. 1996, Lawrence et al. 2006). bFGF has been found in normal
skin, and its level also increases upon injury (Gibran et al. 1994). In animal studies, bFGF appeared to affect several parameters of wound healing rather than a single, specific process and that deficiency causes delayed wound healing (Ortega et al. 1998).

The blister fluid bFGF and IL-10 differed between survivors and non-survivors and between MODS and MOF. It emphasizes the whole body response of severe sepsis with skin involvement as one of the organs affected. The skin is the largest organ and the main defensive barrier; hence the issue of skin failure should be addressed in the context of septic organ failures (Langemo & Brown 2006). Furthermore, other have also suggested that biochemical characterization of wound fluids may be used as biomarkers to reflecting the status of the wound and can even be used as a guidance for treatment interventions (Moseley et al. 2004, Yager et al. 2007). For example, the inhibition of TNF has been shown to both enhance and attenuate skin wound healing. In a mice model, repeated intraperitoneal injections of tumour necrosis factor-binding protein resulted in significantly weaker wounds (Lee et al. 2000). In a septic rat model the breaking strength of incisional wounds was decreased by 40% in septic rats while the administration of a TNF-binding protein to septic rats significantly improved incision wound strength with better collagen organization and deposition (Maish et al. 1998, Rennekampff et al. 2000). In human chronic wounds and in impaired acute wounds, delayed healing has been suggested as being due to excess TNF levels, while topical application of TNF neutralizing antibodies in a murine wound model enhanced wound healing (Ashcroft et al. 2012).

Taken together then, our results show an activated and dysregulated cytokine and growth factor response in the intact skin of severe sepsis patients when compared to the healthy controls. Interestingly, the levels of these mediators in a skin blister wound correlate with severity of the illness as well as the outcome. Further still, the local response did not correlate uniformly with systemic response. This is a novel finding for severe sepsis and can have important implications for wound healing in severe sepsis. The non-existing correlation between plasma and blister fluid levels of some cytokines and other mediators in our series emphasizes the importance of obtaining localized samples whenever studying healing processes. Also, our findings can in the future help develop a method to determine the severity of the disease from blister fluid instead of using different calculation models.
6.2.5 Serum cytokine expression in severe sepsis

Previously, it has been shown that elevated serum levels of IL-6 early in the course of that disease can predict the development of sepsis (Bagdade et al. 2011, Gouel-Chéron et al. 2012). Several studies had suggested IL-6 as a predictor model for sepsis development and its severity (Andaluz-Ojeda et al. 2012, Badiu et al. 2011, Frink et al. 2009). In this current study, IL-6 levels in serum and blister fluid in severe sepsis were elevated compared to the levels in the controls.

The levels of TNF were also elevated in severely septic patients in both blister fluids and serum. This finding is in line with previous studies on the serum levels in peritonitis and sepsis and in the serum of trauma patients with MODS (Badiu et al. 2011, Frink et al. 2009).

Elevated plasma levels of IL-10 have been previously detected in patients with MODS after trauma and also in sepsis (Andaluz-Ojeda et al. 2012, Frink et al. 2009). IL-10 is most often a suppressor of excessive inflammation in sepsis. On the other hand, IL-10 has been proposed as contributing to the compensatory anti-inflammatory response syndrome and death in sepsis (Chiche et al. 2011). This aspect is supported in our series since we found that the non-survivors had higher IL-10 serum levels compared to the survivors, which may be related to immunoparalysis and death in these patients.

In our series, the VEGF levels in severe sepsis were lower in non-survivors than in survivors, but there was no difference noted between septic patients and healthy controls. This finding is in line with the previous study, where VEGF concentrations were lower in nonsurvivors than in survivors, (Karlsson et al. 2008). In the swine wound model, it was found that VEGF is a prominent regulator of the healing process inducing, for example, an earlier appearance of a transforming growth factor beta-1 (Howdieshell et al. 1998).

6.2.6 Skin tight junctions in healing wound and in severe sepsis

In this study the expression of tight junction proteins occludin, claudin-1, claudin-4, and ZO-1 were analysed in healing wounds from the 3rd to 7th day of healing. These wounds were divided into three parts: Intact skin, the hyperproliferative zone, and the leading edge of the migrating keratinocytes.

In intact skin, the tight junction protein expression was very similar in the controls and in the severely septic patients. In the hyperproliferative zone, claudin-1, claudin-4m and occludin expression were similar in severely septic patients and
in the controls. ZO-1 expression was more diffuse and was seen in all epidermal layers compared to the intact skin, and this phenomenon was milder in severe sepsis compared to in the controls. On the leading edge of the migrating keratinocytes claudin-1, ZO-1, and claudin-4 expression was similar in severely septic patients and in the controls. Occludin expression was obvious in the granular cell layer in the severe sepsis group, whereas in the controls, occludin was seen in several cell layers. Also, on the later wounds, occludin was also expressed at the very first point of the leading edge in controls, but not in severely septic patients.

It is well known that in sepsis, tight junctions leak. Nearly in all the organs studied, sepsis has altered tight junction formation or function. In lungs, sepsis affects claudin expression and impairs barrier function causing pulmonary oedema (Koval 2013). In liver, sepsis alters the expression and localization of ZO-1 and occludin and leads to hepatobiliary dysfunction and cholestatic jaundice (Han et al. 2003, Han et al. 2004, Yang et al. 2003). In kidneys, sepsis affects the tight junction gene and protein expression leading to acute kidney failure (Eadon et al. 2012). In the gut, sepsis causes alterations in the TJ architecture and protein distribution, causing oedema and uncontrolled fluid movement (Li et al. 2009). In this study, tight junction expression was quite normal in intact skin and in the healing wound despite a roaring septic storm.

Claudin-1 is very crucial to the barrier function and claudin-1 deficient mice; claudin-6 overexpressing mice die at the early stage due to a massive water loss. From this point of view, it is logical that claudin-1 expression was normal in all samples.

In the acanthotic epidermis of psoriasis, occludin and ZO-1 expression is broadened compared to normally restricted expression (Kirschner et al. 2009, Peltonen et al. 2007). The same effect is seen in the hyperproliferative zone in the healing wound, and in this study, that effort was even more obvious with septic patients than with the controls.

Overall, the tight junctions seem to be very important in several ways, and they remain nearly intact even in severe sepsis. In this study, we only studied the expression of tight junctions proteins, not the function of the tight junctions.
6.3 Ethical aspects of the study

6.3.1 Ethical considerations

This study was carried out in an intensive care unit, which causes some limitations and ethical considerations to the study. Severely septic patients are often sedated and mechanically ventilated and unable to give or deny permission by themselves. This is why the consents in this study were mainly obtained from the next of kin. The patient was able to discontinue the study whenever he/she wanted to, and it was emphasized that a refusal or unwillingness to continue would not affect the care given. Both oral and written informing was given.

We used the suction blister method that familiar to the study group. It is fairly noninvasive and painless model that creates a standardized epidermal wound. No infectious complications were observed even though there was theoretically a chance to blister wound infection in this population. This method has been used for more than 200 patients in different study settings in OUH. Blood samples for the study were collected at the same time as the diagnostic samples, where possible, to avoid extra venous sampling.

Skin biopsies were performed by Koskela and Gäddnäs. Biopsies were taken under local anaesthesia in the intensive care unit using appropriate specimens and sterility. The patients or next of kin was informed about the biopsy and only one biopsy per patient was taken. No wound infections were observed in the biopsy wounds. The healing wounds were excised in the same manner as the biopsies were.

The study data was stored in a safe place according to data registration requirements. Only the study group and two research nurses were allowed to access all of the data.

The ethical board of Oulu University Hospital approved the study plan (Register Number 50/2005).

6.3.2 Methodological considerations

ICU is a demanding environment for carrying out a clinical wound healing study. The method must be rather noninvasive, easy to perform and not cause additional stress for either the patient or the relatives especially during a acute illness.

We used the VapoMeter to measure TEWL. Despite the advantages of the closed chamber system, there were still some limitations. Body temperature affects both water evaporating and blood flow, and some of the patients had hyperthermia
or hypothermia caused by sepsis. The treatments were aimed to restore the normothermia, but the effect of body temperature could not be balanced fully. In this study, there was also no correlation between TEWL and blood flow from the wound and the body temperature.

We decided to use the abdominal skin for suction blister wounds for several reasons. Abdominal skin is accessible, and intact skin is found easily even though some areas were affected or covered. Abdominal skin represents an area with nutritive perfusion, which is less sensitive to body and room temperature changes and does not reflect changes in blood circulation as easy as the arteriovenous areas do (Rendell et al. 1998, Rendell et al. 2000). The Doppler probe of the laser Doppler flow meter was smaller than the wound, so thus the wound value solely represented the blood flow in the wound base. All five suction blisters were measured, and the mean was calculated to reduce the effect of individual differing values in the results.

A Multi-centre study with a bigger study group would improve the generalizability of these results. The suction blister model is fairly arduous and needs some professional experience before starting. To avoid researcher dependent variability, all of the measurements were carried out by Gäddnäs and Koskela. In an intensive care unit setting, it is impossible to standardize the effect of drug therapies or fluid resuscitation. However, we did not find any correlation between the noradrenalin dose or fluid balance and the values measured. All the measurements were performed in the same ICU, so thus the room temperature was likely the same. The patients in this study had severe systemic infections, but the suction blister wounds studied were non-infected.

The same experienced laboratory technician prepared all the histological samples. Samples of the basement membrane components were analysed blindly by two investigators and the other investigator was an experienced dermatopathologist. Tight junction samples were also analysed blindly by two investigators with the other investigator an experienced dermatologist.

The laboratory methods for the cytokine expression of serum and blister fluid expression are standardised and reliable, and all measurements were carried out by experienced laboratory technicians. The non-existing correlation between plasma and blister fluid levels of some cytokines and other mediators in our series emphasizes the importance of obtaining localized samples when studying healing processes.
6.4 Limitations of the study

Even though this study has novel finding it still had some limitations. The study group was heterogeneous and small. It was impossible to rule out all comorbidities and drug therapies. This was a one-centre study. No previous sample size calculation was performed; therefore this study was meant to be experimental and offer valuable information in this setting.

6.5 Clinical importance and future perspectives

Clinically, sepsis has been associated with skin blistering, irritation, and pressure ulcers (Barriere & Lowry 1995, Rico et al. 2002, Sommer et al. 2013). Also, severe sepsis complicates surgery by diminishing wound healing. Severely septic patients often need surgical interventions, and impaired healing can lead to various complications. Better understanding the mechanisms of impaired wound healing for severe sepsis will optimise the timing of the surgical procedures.

Even though we showed delayed wound healing in epidermal wounds in the Study I, we could not explain the whole mechanism for the disruption occurring in the next sub-studies II–IV. As discussed earlier, the observed delay in barrier recovery and probably also in re-epithelisation can result from various aspects, whereas the cytokines and growth factors explain just a bit. These factors behind the phenomena need to be investigated further. In this study the cytokines analysed were limited due to resources and financial standing. In the future, several other cytokines, such as TGFβ subtypes, still need more attention.

Due to the small sample size, the correlations regarding the severity of the disease, SOFA score, and survival did not reach statistical significance. These also remain questions that need to be studied in the future. In addition to investigating the prognostic value of the cytokines of the blister fluid, the most important task is to learn the differences between normal and severely septic patient wound healing and invent a way to impact it still further.

In Study IV only the first day samples were analysed. It would be interesting to gather more samples during the course of the disease, not only in the beginning and if possible, also gather several samples per day. Also a specific wound healing kit for the cytokine examination would be perfect.

It is very unlikely that in the near future every patient in the ICU would have a suction blister to collect blister fluid. The method is usable in study settings but not applicable for everyday or diagnostic use even though the method can give us...
valuable information about the condition of the skin during the disease. However, if we could find the crucial factors that affect wound healing or predict the outcome of the healing process, we could design a precise clinical setting to study these factors. In skin wounds, this process could mean having a tailor-made cocktail of cytokines for a patient with several risk factors for diminished wound healing undergoing major surgery or a cytokine cocktail boost to help a poorly healing wound. In septic animals, inhibition of TNF improves the altered wound healing process (Maish et al. 1998). The administration of a specific TNF antagonist significantly attenuated the effects of sepsis on granulation tissue histology, but not to the control levels (Cooney et al. 1997). More generally, this result could mean, for example, having a laboratory test or small biopsy during a surgical procedure in the operating room on the basis of which a decision to do or not do the anastomoses in bowel resections in the same way as a thromboelastometry is used could be made.

It has also been demonstrated that stress and stress-derived hormone agonists or antagonists (e.g., glucocorticoids, acetylcholine, dopamine, histamine, and catecholamines) have a profound effect on the cutaneous barrier function, wound healing, and the susceptibility to skin infection (Curtis & Radek 2012, Nakatsuji et al. 2013). The skin microbiome is regulated by dermatotopography, as well as the concentration and activity of neuropeptides and endogenous stress hormones that are secreted by resident cells of the epidermis and dermis during stress. Thus, it would be interesting to evaluate the epidermal and dermal microbiome during severe sepsis using the suction blister model. Because the local microbiome is affected by systemic factors, and inflammation plays a key role in the composition of the microbiome, a manipulation of the microbiome to benefit healing after cutaneous injury is also a potential positive target for future research (Holmes et al. 2015).
7 Conclusions

This study offers novel information about skin and wound healing in severe sepsis as follows.

1. The barrier restoration was diminished, and inflammation in the wound was more intense for severe sepsis compared to the controls.
2. The expression of basement membrane components (laminin-332 and Type IV collagen) decreased during the severe sepsis, but increased up until 3 months without achieving the expression level of components in the controls.
3. The expression of the tight junction proteins claudin-1, claudin-4 and ZO-1 remained nearly intact for healing wounds in severe sepsis compared to the controls. The expression of occludin on the leading edge of the migrating keratinocytes was more restricted and late in severe sepsis when compared to the controls.
4. The cytokine profile in severe sepsis differed from the healthy controls in the skin and in the serum. The levels of TNF, IL-10 and IL-6 in skin were higher for severe sepsis compared to controls. The blister fluid and serum cytokine response in severe sepsis differed since the levels of EGF, VEGF, TNF and bFGF in blister fluid did not correlate with the levels of serum. The septic patients with MOF had higher levels of IL-4, IL-10, TNF and bFGF in the blister fluid and IL-10 in serum. Survivors also had lower levels of IL-10 and bFGF in the blister fluid than non-survivors did.

All in all the findings of this study suggest that skin dysfunction in severe sepsis exists even when the most profound structures still remain intact.
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Washburn NR, Prata JE, Friedrich EE, Ramadan MH, Elder AN, and Sun LT Polymer-conjugated inhibitors of tumor necrosis factor-α for local control of inflammation. Biomatter 3(3).


List of original publications


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Original publications are not included in the electronic version of the dissertation.
1383. Raatiniemi, Lasse (2016) Major trauma in Northern Finland
1386. Lappalainen, Olli-Pekka (2016) Healing of cranial critical sized defects with grafts, stem cells, growth factors and bio-materials
1387. Ronkainen, Justina (2016) Role of Fto in the gene and microRNA expression of mouse adipose tissues in response to high-fat diet
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