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Fiia Gäddnäs

INSIGHTS INTO HEALING RESPONSE IN SEVERE SEPSIS FROM A CONNECTIVE TISSUE PERSPECTIVE

A LONGITUDINAL CASE-CONTROL STUDY ON WOUND HEALING, COLLAGEN SYNTHESIS AND DEGRADATION, AND MATRIX METALLOPROTEINASES IN PATIENTS WITH SEVERE SEPSIS

FACULTY OF MEDICINE,
INSTITUTE OF CLINICAL MEDICINE,
DEPARTMENT OF ANAESTHESIOLOGY,
DEPARTMENT OF SURGERY,
DEPARTMENT OF DERMATOLOGY AND VENEREOLOGY,
UNIVERSITY OF OULU

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A longitudinal case-control study on wound healing, collagen synthesis and degradation, and matrix metalloproteinases in patients with severe sepsis

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Abstract

Sepsis is a major challenge for healing responses maintaining homeostasis. Coagulation and inflammation are activated at the whole-body level, even in undamaged tissues. Despite constantly growing knowledge and advances in care, high mortality in severe sepsis remains. It was hypothesised that tissue regeneration processes may also be altered in severe sepsis.

The study population consisted of 44 patients with severe sepsis and 15 healthy controls. Serum samples were obtained during ten days of severe sepsis and twice again, three months and six months later. Experimental suction blisters were performed twice during severe sepsis and at 3 and 6 months. Serum samples were obtained and suction blisters were induced once in controls. Biochemical analyses were performed to assess the level of procollagen I and III aminoterminal propeptides (PINP, PIIINP), reflecting the synthesis of corresponding collagens; in serum and suction blister fluid. In addition collagen I degradationproduct in serum was measured. Physiological measurements of transepidermal water loss and blood flow were done in order to evaluate the re-epithelisation rate and blood flow in an experimental wound. Levels of matrix metalloproteinases (MMPs) 2, 8 and 9 were measured from serum and suction blister fluid.

Decrease in water evaporation from an experimental blister wound was slower in sepsis than in controls. On the fourth day the sepsis patients had higher blood flow in the blister wound than the controls (both in the healing wound and in the newly induced wound). The procollagen III aminoterminal propeptide (PIIINP) levels were increased in serum in severe sepsis, whereas procollagen I aminoterminal propeptide (PINP) levels were not, making up a pronounced PIIINP/PINP ratio. PIIINP and PINP levels were associated with disease severity and outcome. In addition, collagen I degradation measured with ICTP assay was increased in severe sepsis and PINP/ICTP ratio was lower. Furthermore, the overall protein concentration and PINP and PIIINP levels were low in suction blister fluid, which implies that the balance of the extracellular matrix consistence is disturbed in uninjured skin in severe sepsis. Then again in survivors the levels of PINP and PIIINP were up-regulated at three months but returned to normal by six months. MMP-9 levels in serum and skin blister fluid were lower in severe sepsis than in controls during the ten days studied. The MMP-2 levels were found to be increased both in serum and in skin blister fluid in severe sepsis in comparison to the controls and MMP-2 was associated with disease severity and outcome. MMP-8 was increased in serum and in skin blister fluid.

In conclusion, the balance of collagen turnover is altered in severe sepsis in serum and skin and epidermal re-epithelisation is delayed. The levels of MMP-2 and MMP-8 are increased whereas levels of MMP-9 are depressed.

Keywords: collagen, matrix metalloproteinases, procollagen propeptide, Severe sepsis, skin, suction, wound healing

Armo on objektiivisen oivalluksen, uuden uskalluksen ja ymmärryksen lisääntymisen lähtökohta.

F.G. & E.P. 2009

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Abbreviations

ACCP	American College of Chest Physicians
ACE	Angiotensin converting enzym
AKI	Acute kidney injury
ALI	Acute lung injury
alpha-SMA	Alpha smooth muscle antigen
APACHE II	Acute physiology and chronic health evaluation
ARDS	Adult respiratory distress syndrome
ATP	Adenosine triphosphate
CARS	Compensatory anti-inflammatory response syndrome
CGR	Calcitoningene-related peptide
CVP	Central venous pressure
DAMP	Damage associated molecular patterns
DIC	Disseminated intravascular coagulation
ECM	Extracellular matrix
ECP	Eosinophil chemotacticprotein
EGF	Epidermal growth factor
ET	Endothelin
FACIT	Fibril associated collagens with interrupted triple helices
FGF	Fibroblast growth factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
ICAM	Intracellular adhesion molecule
ICTP	Type I collagen cross-linked telopeptides
ICU	Intensive care unit
IGF	Insulin-like growth factor
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
LT	Leukotriene
MAP	Mean arterial pressure
MCP	Monocyte chemotacticprotein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
mRNA	Mitochondrial ribonucleic acid

MT-MMP	Membrane type Matrix metalloproteinase
NF-κβ	Nuclear factor kappa beta
NO	Nitric oxide
OPS	Orthogonal polarized spectral
PAF	Platelet activating factor
PAMP	Pathogen associated molecular patterns
PAR	Pressure adjusted heart rate
PAR	Protease activated receptor
PDGF	platelet-derived growth factor
PECAM-1	Platelet endothelial adhesion molecule 1
PG	Prostaglandin
PICP	Procollagen type I carboxyterminal propeptide
PIIINP	Procollagen type III aminoterminal propeptide
PINP	Procollagen type I aminoterminal propeptide
PIRO	Predisposition Insult/infection Response and Organ failure
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAPS	Simplified acute physiology score
SCCM	Society of critical care medicine
SDF	Side stream dark field
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment
TGF-beta	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metalloproteinases
TLR	Toll-like receptor
TNF-alpha	Tumor necrosis factor alpha
TNM	tumour nucleus metastasis
TXA	Thromboxane
VCAM	Vascular endothelial adhesion molecule
VE-cadherin	Vascular endothelium cadherin

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition the thesis includes unpublished results.

I Gäddnäs F, Koskela M, Koivukangas V, Risteli J, Oikarinen A, Laurila J, Saarnio J & Ala-Kokko T (2009) Markers of collagen synthesis and degradation are increased in serum in severe sepsis: a longitudinal study of 44 patients. Critical Care 13: R53.

II Koskela M*, Gäddnäs F*, Ala-Kokko T, Laurila J, Saarnio J, Oikarinen A & Koivukangas V (2009) Epidermal wound healing in severe sepsis and septic shock in humans. Critical Care 13: R100.

III Gäddnäs F, Koskela M, Koivukangas V, Laurila J, Saarnio J, Risteli J, Oikarinen A & Ala-Kokko T (in press) Skin collagen synthesis is depressed in patients with severe sepsis. Anestesia and analgesia.

IV Gäddnäs F, Sutinen M, Koskela M, Tervahartiala T, Sorsa T, Salo T, Laurila J, Koivukangas V, Ala-Kokko T* & Oikarinen A* (2010). Matrix metalloproteinases 2, 8 and 9 in serum and skin blister fluid patients with severe sepsis. Critical Care 14: R49.

*with equal contribution

Contents

1 Introduction

Sepsis is a major challenge for healing responses that maintain homeostasis. Coagulation, inflammation and tissue regeneration processes are activated at the whole-body level (Marshall 2001). Despite growing understanding of pathophysiological processes in sepsis and improvements in supportive care and pharmacological therapies, the disease often leads to dysfunction of multiple organs and high mortality (Brun-Buisson 2000, Angus *et al.* 2001, Martin *et al.* 2003, Harrison *et al.* 2006).

The extracellular matrix (ECM) provides structural support to tissues. Constant remodelling and cellular signalling take place in order to maintain homeostasis in steady state, but also in development and disease. ECM consists of fibrous collagens and elastins and other structural and adhesive proteins like fibronectin and laminin in a hydrated polysaccharidegel of glycosaminoglycans. Collagens I and III are the most abundant proteins of ECM and are produced by fibroblasts (Risteli & Risteli 2006). Excessive collagen accumulation in organs is termed fibrosis.

Sepsis patophysiology is most profoundly studied in coagulation and inflammation abnormalities, but the repair processes of tissues are poorly understood. Adult respiratory distress syndrome (ARDS) is one manifestation of organ failure in severe sepsis. Studies on ARDS have revealed that fibrosis occurs much earlier than previously thought, already during the first day, and it impacts outcome (Clark *et al.* 1995, Marshall *et al.* 2000b). The processes that lead to progressive fibrosis and dysfunction in ARDS, and on the other hand processes that make adaptive fibrotic repair response possible, need to be mapped out and developed to therapies. Furhermore, in other organs, the role of ECM remodelling (along with parenchymal cell regeneration) in tissue repair may open new insights to therapeutical approaches.

Previous studies have not examined markers of collagen synthesis and degradation on the systemic level in human severe sepsis. In patients with multiple organ failure with traumatic etiology the serum procollagen III levels are increased with poor prognosis (Waydhas *et al.* 1993). In addition to studying serum levels of markers of collagen synthesis and degradation, we designed to examine whether synthesis of collagens is altered in skin that serves as the main barrier in host defence to external pathogens. In addition, healing of experimental epidermal wound and skin microcirculation in sepsis was studied. The balance between synthesis and degradation make up the turnover of collagen. In the fourth

study, the levels of collagen degrading matrix metalloproteinases were studied. The studied MMPs -2, -8 and -9 have, additive to ECM degrading functions, interesting roles in inflammatory processes such as the massive systemic inflammatory process in sepsis. These studies give insights into the role of extracellular matrix remodelling in the pathophysiology of multiple organ failure in sepsis at systemic and organ levels.

2 Rewiew of the literature

2.1 Collagens

2.1.1 Classification

The protein family of collagens form the main constituent of extracellular matrix and provide structural support to the organs. The number of the family members in vertebrates is at least 27 and is constantly growing (Myllyharju & Kivirikko 2004). A collagen molecule includes three polypeptide chains, called α-chains, with repeated Glycine-X-Y sequence. Collagens can be divided into nine distinct families according to the supramolecular structures and other properties they share (Table 1.). Collagen hybrid molecules and alternative splicing of the transcripts as well as alternative use of promoter regions further add to the heterogeneity of collagens (Myllyharju & Kivirikko 2004).

2.1.2 Fibrillar collagens

The group of fibrillar collagens consists of collagen types I, II, III, V, XI, XXIV and XXVII and they provide structural support to the organs (Myllyharju & Kivirikko 2004). In mammals, type I collagen forms approximately 70% of total collagen, whereas type III accounts for 5 to 20% (Adachi *et al.* 1997). Fibrillar collagens share the unique ability to form non-interrupted triple helices that self-assemble into fibrils and fibres by forming intra- and intermolecular cross-links. Thecollagen fibre has a stabile structure that is resistant to cleavage with most of the proteinases.

Table 1. Collagen types, genes, molecular forms and distribution in human tissues. (Modified from (Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004)).

Type	Subgroup	Gene	Molecular forms/gene product	Distribution
I	Fibrillar	COL1A1	[α1(I)]2α2(I)	Most tissues
		COL1A2	[α1(I)]3	
II	Fibrillar	COL2A1	[α1(II)]3	Cartilage, cornea, vitreous humor, intervertebral disc
III	Fibrillar	COL3A1	[α1(III)]3	Soft tissues, with type I collagen
IV	Network-forming	COL4A1	[α1(IV)]2α2(IV)	Basement membranes
		COL4A2	[α3(IV)]2α4(IV)	
		COL4A3	other forms	
		COL4A4		
		COL4A5		
		COL4A6		
V	Fibrillar	COL5A1	[α1(V)];3	Minor amounts in most tissues with type I collagen
		COL5A2	α1(V)α2(V)α3(V)	
		COL5A3	other forms	
VI	Beaded filament-forming	COL6A1	α1(VI)α2(VI)α3(VI)	Minor amounts in most tissues
		COL6A2		
		COL6A3		
VII	Anchoring fibril-forming	COL7A1	[α1(VII)]3	Skin, cervix, oral mucosa
VIII	Network-forming	COL8A1	[α1(VIII)]2α2(VIII)	Many tissues
		COL8A2		
IX	FACIT	COL9A1	α1(IX)α2(IX)α3(IX)	With type II collagen, e.g. cartilage
		COL9A2		
		COL9A3		
X	Network-forming	COL10A1	[α1(X)]3	Hypertrophic cartilage
XI	Fibrillar	COL11A1	α1(XI)α2(XI)α1(II)	With type II collagen e.g. cartilage
		COL11A2	other forms	
		COL2A1		
XII	FACIT	COL12A1	[α1(XII)]3	Many tissues with type I collagen
XIII	Transmembrane domain	COL13A1	Unknown	Minor amounts in many tissues
XIV	FACIT	COL14A1	[α1(XIV)]3	Many tissues with type I domain
XV	MULTIPLEXINs	COL15A1	Unknown	Many tissues
XVI	FACIT	COL16A1	[α1(XVI)]3	Many tissues
XVII	Transmembrane domain	COL17A1	[α1(XVII)]3	Hemidesmosomes of stratified squamous epithelia
XVIII	MULTIPLEXINs	COL18A1	α1(XVIII)	Liver, kidney, placenta, etc.
XIX	FACIT	COL19A1	α1(XIX)	Several tissues

Type	Subgroup	Gene	Molecular forms/gene product	Distribution
XX	FACIT	COL20A1	α1(XX)	Corneal epithelium, skin, cartilage and tendon
XXI	FACIT	COL21A1	α1(XXI)	Many tissues
XXII	FACIT	COL22A1	α1(XXII)	Tissue junctions
XXIII	Transmembrane domain	COL23A1	α1(XXIII)	Metastatic tumour cells
XXIV	Fibrillar	COL24A1	α1(XXIV)	Developing bone and cornea
XXV	Transmembrane domain	COL25A1	α1(XXV)	Neurons
XXVI	FACIT	COL26A1	α1(XXVI)	Testis, ovary
XXVII	Fibrillar	COL27A1	α1(XXVII)	Cartilage, eye, ear and lung

2.1.3 Biosynthesis of fibrillar collagens

The main cells synthesizing fibrillar collagens are fibroblasts. Also, smooth muscle cells are capable of synthesizing collagens including types I and III. The α-chains are synthetised on the ribosomes of the endoplasmic reticulum from mRNAs transcripted from procollagen genes. After the cleavage of the signal peptides from the aminoterminal end, there are many post-translational modifications, for which several specific enzymes are required. Collagen prolyl 4-hydroxylase and prolyl 3-hydroxylase account for hydroxylation of proline residues in X and Y positions. As a result 3-hydroxyproline and 4-hydroxyproline residues are formed. Lysyl hydroxylase hydroxylates lysine residues in Y positions to hydroxylysine.

Some hydroxylysyl residues are further glycosylated to galactosylhydroxylysine and glucosylgalactosylhydroxylysine. After these modifications to the α-chains, the C-propeptides of three chains are directed by recognition sequences to from inter- and intramolecular disulfide bonds. After the C-propeptides have become associated, a triple helix is formed in a zipper like fashion towards the N-terminus.

The resulting procollagen molecules are packed in the Golgi apparatus to be carried to the cellular membrane and excreted to the extracellular space. Extracellularly the carboxy- and aminoterminal propeptides are cleaved off by N- and C- proteinases. The remaining triple-helix is self-assembled into collagen fibril by nucleation, propagation and formation of covalent cross-links and further

arranged to collagen fibres by intermolecular cross-links. (Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004)

2.1.4 Procollagen propeptides of types I and III collagens as markers of collagen synthesis

During the biosynthesis of collagen, types I and III carboxy and aminoterminal propeptides are cleaved from a procollagen molecule in equimolar amounts with collagen molecules and thus reflect directly the synthesis rate of collagen (Risteli & Risteli 2002). Radioimmunological assays have been developed and are commercially available for type I procollagen carboxy- and aminoterminal propeptides (PICP, PINP respectively) and for type III procollagen aminoterminal propeptide (PIIINP) (Risteli *et al.* 1988, Melkko *et al.* 1990, Melkko *et al.* 1996). The 95% reference interval for PINP is 20–76 µg/L for men and 19–84µg/L for women (Melkko *et al.* 1996), whereas that of PIIINP is 1.7–4.2 µg/L for adults (Risteli *et al.* 1988). Once the propeptides are released, they find their way to the circulation without being further metabolized in situ. The propeptides from soft tissues pass through the lymphatic drainage whereas propeptides from bone pass directly to circulation. Consequently, the concentration of PIIINP, mainly synthetized in soft tissues, is ten times higher in lymph than in serum, whereas PINP, the main constituent of bone ECM, is equal in these body liquids (Jensen *et al.* 1990). There are some factors causing variation in serum measurements. For instance the cleavage of PINP can be delayed, which results in disproportionate increase in PICP. On the other hand, the differences in elimination are seen as lower PICP/PINP ratio, particularly in children (Tähtela *et al.* 1997).

PICP and PINP

The type I procollagen molecule consists of the collagen proper molecule and the carboxy and aminoterminal propeptides (PICP, PINP) (Figure 1). The most abundant molecular form of collagen I consists of two α1(I) chains and one α2(I) chain. Other forms also exist (Table 1). In both ends of the triple helix there is a short noncollagenous telopeptide region essential for cross-linking the chains together.

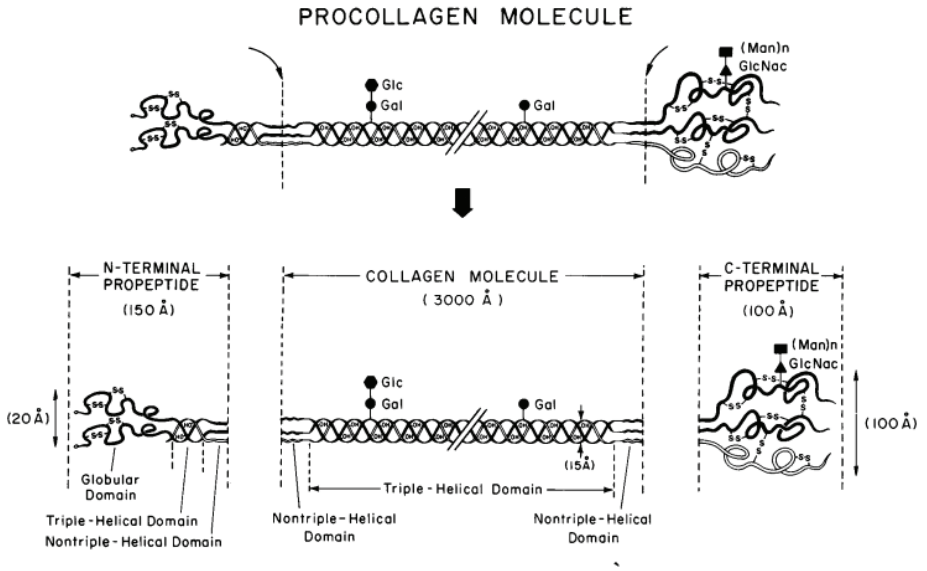

Fig. 1. A schematic presentation of the structure of a typical procollagen I molecule containing two α1(I) chains and one α2(I) chain. Glc, glucose, Man, Mannose, GlcNac, N-asetylglucosamine (Reprinted by permission from (Prockop *et al.* 1979) (Copyright © [1999] Massachusetts Medical Society. All rights reserved.)).

PICP is a globular trimeric structure with molecular weight of 100 000 daltons.

In blood the antigenic structure of PICP is the one of the authentic propeptide (Melkko *et al.* 1990). The circulating PICP is eliminated by mannose receptors on liver endothelial cells (Smedsrod *et al.* 1990). Elimination via kidneys does not occur, as the molecule is too large.

The structure of PINP consists of three domains: Globular aminoterminal, triple helical collagenous and short non-collagenous domains that form a loosely rod-like elongated structure. The latter links the propeptide to collagen proper. The molecular weight of PINP is 35 000 daltons. In the circulation two different antigenic structures for PINP are found. The first is identical to the trimeric authentic propeptide, and the second has the size of the globular aminoterminal domain. The smaller antigenic structures seem to be derived from uncleaved forms of PINP that remain in tissues or from degradation of uncleaved procollagen. Thus the most sensitive measurement of collagen synthesis is achieved by measuring the intact PINP only (Risteli & Risteli 2006). The acidic nature of PINP is advantageous when isolating it from body fluids containing high concentrations of propeptide, e.g. pleural effusion or ascites (Melkko *et al.*

1996). Elimination of the PINP molecule occurs also by the liver endothelial cells, but via scavenger receptor (Melkko *et al.* 1994). It is notable that only liver damage affecting the endothelial cells can alter the elimination rate. Smaller peptides, such as the globular aminoterminal domain of the PINP, can be eliminated via kidneys.

The procollagen III molecule contains carboxy and aminoterminal propeptides with the homotrimeric (three α1(III)chains) collagen III molecule in between. PIIINP is structurally close to PINP containing three domains. The antigenic variability in blood is even more complex than for PINP due to a transglutaminase site, which enables cross-linking with another PIIINP molecule or other connective tissue components. The specifity of the different PIIINP assays in detecting only authentic PIIINP and its possible dimers varies. The test used for analysis in the current studies is the most specific so far (Orion diagnostica, Espoo, Finland). PIIINP is eliminated via liver endothelial cell scavenger receptors such as PINP, and only smaller fragments similar to the size of the globular aminoterminal domain can be eliminated via kidneys. (Risteli & Risteli 1995)

2.1.5 Collagen degradation

Fibrillar collagens are degradaded trough two distinct parthways, intracellular and extracellular. In the intracellular pathway, cytoplasmic protrusions move around the collagen fibril, forming a phagosome that separates the fibril from extracellular matrix. When lysosomes fuse to the phagosome, the lysosomal enzymes degrade the collagen fibril. TGF-β seems to increase phagosytosis, while IL1α inhibits it (van der Zee *et al.* 1997).The extracellular pathway is used during excessive breakdown of collagen as seen in inflammatory diseases. (Song *et al.* 2006)

The proteases participating in the breakdown of fibrillar collagens can be roughly divided into three groups: matrix-metalloproteinases, cathepsins and various unspecific proteinases that are able to cleave telopeptides and denaturated collagen, but have less affinity to the native triple helix than specific members in the two former classes.

2.1.6 Matrix metalloproteinases

Structure

In 1962 Gross and Labiere identified collagenase in tadpole tails, an enzyme that degraded fibrillar collagen (Gross & Lapiere 1962). Since then, altogether over 25 structurally related proteinases have been found. This protein family is called matrix metalloproteinases, of which 24 are found in mammals. All mammalian MMPs have similar catalytic domain with autoinhibitory pro-domain. Some of them also contain a hemopexin domain.

The prodomain contains a cysteine residue with sequence whose sulphydryl moiety forms a non-covalent bond with the zinck-ion (Zn^{2+}) at the active site. The active site contains a Met residue and two Zn^{2+}. One of the Zn^{2+} is needed for the proteolytic activity. The hemopexin group is a four-bladed β-propeller structure that contributes to substrate recognition, activation of the enzyme, protease localization, internalization and degradation. (Maskos 2005) (Figure 2). MMP-2 and -9, also called gelatinases, have fibronectin type II repeats with which they bind to their ligands. MMP-2 is a 72 kDa gelatinase and is widely expressed by various cells whereas 92 kDa MMP-9 is expressed mainly by inflammatory cells such as PMNs, macrophages and myofibroblasts.

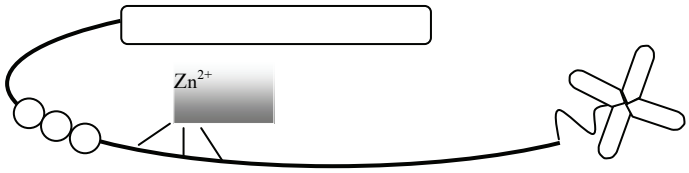

Fig. 2. Most of the MMPs share a conserved structure including a prodomain, a catalytic domain, and a hemopexin domain with a hinge connecting it to the former. The cleavage of the prodomain reveals the active Met-residue with zinc ion. Only MMP-2 and MMP-9 have fibronectin type II repeats included in the catalytic domain.

Classification

The family of MMPs can be classified to subgroups according to substrate specifity and molecular structure (Table 2).

Table 2. Classification and nomenclature of matrix metalloproteinases (Modified from (Viappiani *et al.* 2006, Visse and Nagase 2003)).

Subgroup	MMP nro	Other names
Collagenases	1	Collagenase1/ Interstitial collagenase
	8	Collagenase-2/ Neutrophil collagenase
	13	Collagenase-3
Gelatinases	2	Gelatinase A
	9	Gelatinase B
Stromelysins	3	Stromelysin-1
	10	Stromelysin-2
	11	Stromelysin-3
Matrilysins	7	Matrilysin-1
	26	Matrilysin-2
Membrane type	14	MTP-1 MMP
MMPs (MT-MMPs)	15	MTP-2 MMP
	16	MTP-3 MMP
	17	MTP-4 MMP
	24	MTP-5 MMP
	25	MTP-6 MMP
Unclassified	12	Metalloelastase
	19	RASI-1
	20	Enamelysin
	21	
	22	
	23	CA-MMP
	27	
	28	Epilysin

Function

Matrix metalloproteinases are best known as enzymes that degrade ECM structural components, especially collagens and proteoglycans. Due to the highly stabile structure of fibrillar collagens, only a limited number of MMPs are able to initiate the cleavage under physiological conditions of 37 °C and neutral pH. These include the group of collagenases, MMP-2 and MMP-14 (MT1-MMP). They cleave collagens I, II and III at specific Gly-Ile bonds in the α1-chain and Gly-Leu-bonds in the α2 chain. As a result ¾ N-terminal and ¼ C-terminal fragments are formed that are susceptible to further degradation by gelatinases and other proteinases. (Song *et al.* 2006) In addition to collagens, matrix metalloproteinases cleave other ECM substrates.

The consequences of cleavage of ECM substrates is not restricted to shedding of the molecules aiming at tissue resorption. In addition to affecting cellular functions by regulating the ECM environment within which and with which the cells interact, the MMPs create space for the migration of inflammatory cells and modify the activity of signalling molecules. For example, cleavage of type I collagen by MMP-1 enables keratinocyte migration during re-epithelization (Pilcher *et al.* 1997) and shedding of type IV collagen of basement membranes, and endothelial tight junction components by gelatinases seems to contribute to enhanced vascular permeability in dengue hemorrhagic shock and blood brain barrier. (Luplertlop *et al.* 2006, Reijerkerk *et al.* 2006) In addition to the degradation of structural proteins of ECM, the MMPs have various non-ECM substrates, the cleavage of which results in various biological activities including the various effects on inflammation and immunity. A detailed review of the plenary of substrates and functions has been provided by Visse and Nagase (2003).

Matrix metalloproteinases modulate inflammation and immunity responses

The roles of matrix metalloproteinases in regulation of inflammation and immunity have become evident during the past decades. MMPs serve as proinflammatory as well as anti-inflammatory regulators. Table 3 summarizes some of the various known functions of MMPs in inflammatory response. In a mouse model of sepsis MMP-9 null mice showed increased dissemination of infection, higher peritoneal cytokine and chemokine levels, diminished recruitment of leukocytes and more severe organ failure (Renckens *et al.* 2006). On the other hand MMP-9 deficiency protects from lethal endotoxin shock (Dubois *et al.* 2002). In a baboon model of E.coli induced sepsis, the MMP-9 levels were up-regulated during the first 24 hours (Paemen *et al.* 1997). In humans, elevated levels of MMP-8 (Hästbacka *et al.* 2007), MMP-9, TIMP-1 and TIMP-2 (Nakamura *et al.* 1998, Hoffmann *et al.* 2006) have been reported on the first days of severe sepsis.

Table 3. The functions of different MMPs in inflammation.

Biological effect	Responsible MMPs	Substrate cleaved	Reference
Modulation of barrier function			
Decreased endothelial cell attachment.	MMP-7	E-cadherin	(Ichikawa *et al.* 2006)
Diapedesis of monocytes	Possibly MMP-2 and	Occludin of the tight	(Reijerkerk *et al.* 2006)

through the blood brain barrier	MMP-9	junctions	
Decreased vascular contractility	MMPs	Not known	(Lalu *et al.* 2006)
Enhanced vascular permeability	MMP-2, -9	VE-cadherin, PECAM-1	(Luplertlop *et al.* 2006)
Chemokine processing			
Potentiation of chemokine signal	MMP-9, -8	IL-8, CXCL6, CXCL5	(Van den Steen *et al.* 2000, Van Den Steen *et al.* 2003)
Attenuation of chemokine signal	MMP-2	CCL7	(McQuibban *et al.* 2002)
Controlling cytokines and growth factors			
IL-1β processing from precursor, proinflammatory	MMP-1, -3, -9	IL-1β	(Schonbeck *et al.* 1998)
IL-1β degradation, anti-inflammatory	MMP-1, -2, -3, -9	IL-1β	(Ito *et al.* 1996)
Increased activity of TGF-β	MMP-2, -9	Latent TGF-β	(Yu & Stamenkovic 2000)
Increased bioavailability of IGF-1 and cell proliferation	MMP-1, -2, -3	IGFBP-1	(Fowlkes *et al.* 1994)
Activation of VEGF	MMPs	CTGF	(Hashimoto *et al.* 2002)
Tissue regeneration and remodelling			
Re-epithelization of injured lung epithelium	MMP-7	VE-cadherin	(McGuire *et al.* 2003)
Keratinocyte migration and re-epithelization	MMP-1	Type I collagen	(Pilcher *et al.* 1997)
Increased inflammation and delayed wound healing	MMP-8 null mice	Not known	(Gutierrez-Fernandez *et al.* 2007)

Mechanisms of activation and inhibition

Generally, MMPs can be activated in two ways (Fig 4). The activation occurs when the active site is revealed by disrupting the connection between the thiol-group in the prodomain and Zn^{2+} in the active site. This can be achieved by the cleavage of the prodomain or conformational change caused by thiol- Zn^{2+} linkage disrupting agents. (Springman *et al.* 1990)

One of the best described proteolytic activation pathway of an MMP by another one is the activation of MMP-2 by the membrane bound MMP-14 (MT1-MMP) complexed with TIMP-2 (Itoh *et al.* 2001). The findings that MMP-2 activation is inhibited in TIMP-2 null mice, but not in MMP-14 null mice suggests that other membrane bound proteinases can participate in MMP-2 activation. Plasmin has been shown to be able to activate, in addition to other

MMPs, MMP-1 in interaction with urokinase and stromelysin-1 in cultured skin cells (He *et al.* 1989). Still, compelling in vivo evidence on the role of plasmin as MMP- activator is lacking. Furin is an intracellular serin protease, and MMPs containing furin cleavage site between pro-and catalytic domains are processed intracellularly. (Fu *et al.* 2008)

Reactions with allosteric molecules or reactive oxygen species can disrupt the thiol-Zn^{2+} linkage and result in active conformation. Also, SDS/APMA used in MMP zymography results in such conformational change. In vivo it is generally considered that this is only a transitional active state that allows autolytic cleavage to active MMP. Yet exceptions have been described. It is suggested for instance that proMMP-1 docked to collagen binding α2β1-integrin is active without removal of the prodomain (Dumin *et al.* 2001). It is hypothetized that other MMP anchoring molecules could also modulate the activity of MMPs. The role of oxidants seems to be important especially in inflammation. Leukocyte derived oxidants have been shown to activate proMMPs-1,-7 and-9 (Weiss *et al.* 1985, Peppin & Weiss 1986, Fu *et al.* 2001), and peroxynitrite generated by interaction of nitric oxide and superoxide can activate MMP-8 (Okamoto *et al.* 1997).

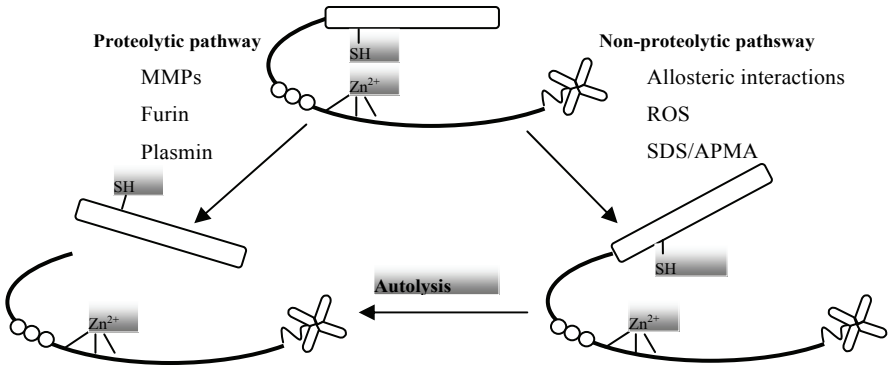

Fig. 3. Mechanisms of MMP activation. For activation the thiol-Zn^{2+} interaction needs to be disrupted, an event termed the "cysteine-switch". Two main activation pathways have been described. The first is activation by cleavage of the inhibitory prodomain by MMPs or other proteinases. The second pathway to activation is via reactions, where only disruption of thiol-zinc interaction occurs. In vivo autolytic proteolysis of the prodomain is considered to be the final step in activation, although examples of active MMPs containing the prodomain exist.

Hence, there seems to be multiple alternative pathways of MMP-activation. In addition to the concentration of an active enzyme, substrate specifity and the cellular environment also affect the level of activity observed. It is generally accepted that there is considerable overlap between the substrates different MMPs cleave, although the affinity to specific substrates is stronger. Hence the amounts of more potent substrates available partly control the activity on other substrates. (Fu *et al.* 2008)

MMP activating agents

MMPs participate in tissue remodelling and thus it is not surprising that tissue injury upregulates their activities. Skin injury has been shown to enhance MMP-2 expression and activity (Jansen *et al.* 2007). The proinflammatory cytokine, tumor necrosis factor alpha (TNF-α) as well as transforming growth factor beta (TGF-β) have been shown to induce MMP-2 and MMP-9 expression and activity (Han *et al.* 2001a, Han *et al.* 2001b).

MMP inhibiting agents

Tissue inhibitors of matrix metalloproteinases are a group of four endogenous glycoproteins (TIMP-1,-2,-3,-4) that inhibit MMP activity in vitro. Direct evidence of inhibitory function in vivo is not yet compelling (Fu *et al.* 2008). Plasma α-macroglobulins are unspecific endopeptidase inhibitors shown to be able to trap active MMPs and mediate their uptake by macrophage scavenger receptors. Also other endogenous proteins are able to inhibit specific MMPs. (Visse & Nagase 2003)

2.1.7 Cathepsins

The cathepsin family consists of 16 proteinases with subgroups of serine-, aspartic-, and cysteineproteinases. Most of them are lysosomal endoproteinases, except cathepsins E and G, and function in acidic pHs intracellularly. However, cathepsin K is able to cleave collagen I still in pH 5.0–6.0. It is able not only to cleave collagens in the telopeptide regions as cathepsins usually do, but also at various sites in the triple helical region. It is the key enzyme in bone collagen degradation. Localized at the ruffled cell border in the osteoclasts it is released to the acidic reserption lacunae to degrade bone matrix proteins (Song *et al.* 2006).

2.1.8 ICTP in measuring collagen degradation

Before more accurate measurements assays for collagen degradation were developed, urine 4-hydroxyproline was used as a measure of collagen degradation. The limitations of this assay are that part of hydroxyproline is dietary and a part is released from other proteins than collagen, i.e. C1q component of the complement system. In addition, only 10% of hydroxyproline is excreted, and a part of it is derived from the aminoterminal propeptides during the synthesis of collagen. At present, more accurate assays that detect cross-links in the telopeptide areas are available. These cross-link antigens can be only derived from degradation of collagen fibres and thus are more accurate measures than hydroxyproline assays. Yet these cross-links exist in several collagen types and thus are unspecific. The next step in development was to develop assays detecting specific peptide regions containing cross-links in telopeptide regions of collagen I. The antigens of CrossLaps and ICTP assays are presented in Figure 4. (Risteli & Risteli 2002)

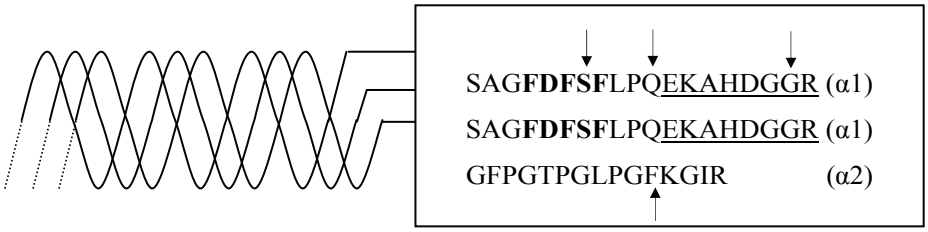

Fig. 4. Antigenic structures recognised by CrossLaps and ICTP assays in the collagen I telopeptide. The ICTP assay detects two adjacent phenylalanine rich FDFSF (bold) domains. Such a structure is possible only when there is a trivalent crosslink. The ICTP assay also detects α1 homotrimer forms of collagen I. The Cross Laps assay detects an aminoacid sequence EKAHDGGR (underlined). For β-β CrossLaps analysis two cross-linked sequences are required. The arrows mark the cathepsin K cleavage sites (modified from (Garnero *et al.* 2003, Sassi *et al.* 2000)).

The CrossLaps assay detects aspartic acid that can be either in α or β isoform. Additionally, because the antigen is one of six amino acids the assay only detects uncross-linked variants in addition to di-and trivalently cross-linked. To overcome these problems β-β-assay, which requires two adjacent sequences, has been developed. Furthermore, possibility to L to D racemisation of the epitope has complicated the assay development.

Collagen degrading enzymes have specific cleavage points in the telopeptide region, which accounts for the fact that the CrossLaps assay measures cathepsin K-mediated bone collagen degradation and ICTP detects telopeptides cleaved by matrix-metalloproteinases (Garnero *et al.* 2003). Since cathepsin K is able to cleave the ICTP epitope (Figure 4), the ICTP assay underestimates degradation in situations where collagen is exposed to active cathepsin K (Garnero *et al.* 2003). Matrix metalloproteinases are, apart from membrane bound MMPs, exopeptidases, whereas cathepsins function mainly in intracellular vacuoles. Hence measuring ICTP gives insights to extracellular collagen degradation that mainly occurs in situations of pathologically increased collagen turnover. Since the skeleton is the main source of collagen I degradation products, the question whether the altered ICTP levels are a result of a specific disease or due to immobilization caused by the disease, remains an issue to be discussed. Developing an assay measuring degradation of collagen III would be beneficial for evaluating the degradation in soft tissues (Risteli & Risteli 1995). The 95% reference interval for ICTP in serum in adult population is 1.6–4.6 µg/L (Risteli *et al.* 1993).

2.2 Definition and epidemiology of severe sepsis

Mortality in sepsis is high in Finland as well as in other countries, despite the advances in antimicrobial and organ supportive therapies. In Finland the incidence of severe sepsis requiring ICU treatment is 0.38/1000 adults per year and the one year mortality rate is 40.9% (Karlsson *et al.* 2007). According to this incidence figure, in our University hospital district area approximately 200 adults are treated for severe sepsis annually. In the United States both the incidence and mortality in severe sepsis are increasing. The figures were 1.32/1000 and 49.7%, respectively in 2003. The hospitalization rate increases approximately 8%/year and population based mortality rate 6%/year, and are associated with increasing age (Dombrovskiy *et al.* 2007). In a European study the overall proportion of patients with severe sepsis in ICU admissions was 30% (930/3147 patients), although varied in different countries (Vincent *et al.* 2006).

The nomenclature of sepsis is somewhat varied and terms like septicaemia, sepsis and sepsis syndrome have been used without precise definitions. The 1992 Concensus conference of the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) published definitions for sepsis, severe sepsis and septic shock and these criteria have been internationally accepted (Bone *et al.* 1992). Systemic inflammatory response syndrome (SIRS) is a term used to describe systemic activation of immune response regardless of the cause. Sepsis is further defined as SIRS with suspected or microbiologically evident infection. (Table 4).

Table 4. The SIRS criteria (Bone *et al.* 1992).

SIRS is present with ≥ 2 of the following symptoms
Temperature > 38 ºC or < 36 ºC
Heart rate > 90 beats per minute
Respiratory rate > 20 breaths per minute or pCO2 < 32 mmHg (4.3 kPa)
White blood cell count > 12 × 109/l or < 4 × 109/l or > 10% immature (band) forms

New insights into sepsis patophysiology and development of diagnostic tests generated a need to revisit the criteria. Several North American and European intensive care societies convened to specify the criteria (Levy *et al* 2003). The sepsis criteria were reinforced with some clinical and laboratory parameters as well as findings indicative of early organ failure. Some of these and traditional SIRS parameters in the presence of documented or suspected infection were

included in the new definition, mainly serving a clinician rather than creating categorical criteria useful for research work (Levy *et al* 2003). The definition of severe sepsis remained the same. The term "severe sepsis" is used when sepsis is complicated with organ dysfunction (Figure 5). The organ failures can be determined using Multiple Organ Dysfunction score developed by Marshall *et al.* (1995) or by using the Sequential Organ Failure Assesment (SOFA) (Vincent *et al.* 1996, Vincent *et al.* 1998) (Tables 5 and 6).

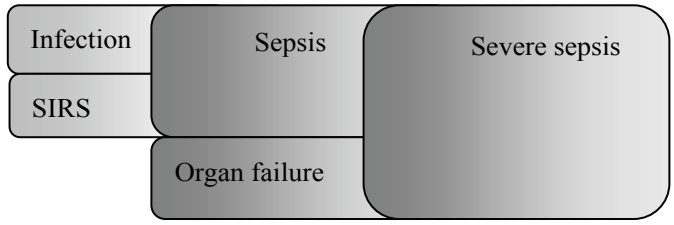

Fig. 5. The relationships within sepsis terminology.

Table 5. Multiple organ dysfunction score (Marshall *et al.* 1995).

Organ system	Multiple organ dysfunction score				
	0	1	2	3	4
Respiratory					
PaO_2/FiO_2 (mmHg)	> 300	226–300	151–225	76–150	≤ 75
Renal					
Creatinine (µmol/L)	≤ 100	101–200	201–350	351–500	> 500
Hepatic					
Bilirubin (µmol/L)	≤ 20	21–60	61–120	121–240	> 240
Cardiovascular *(PAR)*	≤ 10.0	10.1–15.0	15.1–20.0	20.1–30.0	> 30
Coagulation					
Platelets (10^3/µL)	> 120	81–120	51–80	21–50	≤ 20
Neurologic					
Glascow Coma Score	15	13–14	10–12	7–9	≤ 6

PAR, the Pressure adjusted heart rate [PAR = heart rate*(CVP/MAP)], where CVP is central venous pressure and MAP mean arterial pressure.

Table 6. Sequential organ failure assessment (Vincent *et al.* 1996, Vincent *et al.* 1998).

Variables	SOFA Score				
	0	1	2	3	4
Respiratory					
PaO_2/FiO_2 (mmHg)	> 400	≤ 400	≤ 300	≤ 200 With respiratory support	≤ 100 With respiratory support
Coagulation					
Platelets ($10^3/\mu L$)	≥ 150	< 150	< 100	< 50	< 20
Liver					
Bilirubin (mg/dL)	< 1.2	1.2–1.9	2.0–5.9	6.0–11.9	> 12.0
(μmol/L)	< 20	20–32	33–101	102–204	> 204
Cardiovascular					
Hypotension	No hypotension	MAP < 70mmHg	Dopamine ≤ 5 or dobutamine any dose	Dopamine > 5 or epi ≤ 0.1 or norepi ≤ 0.1	Dopamine> 15 or epi > 0.1 or norepi > 0.1
Central nervous system					
Glascow Coma Score	15	13–14	10–12	6–9	< 6
Renal					
Creatinine (mg/dL)					
(μmol/L)	< 1.2	1.2–1.9	2.0–3.4	3.5–4.9	> 5.0
or urine output (mL/d)	< 110	110–170	171–299	300–440 < 500	> 440 < 200

epi, epinephrine; norepi, norepinephrine; Adrenergic agents administered for at least 1hr (doses given are in μg/kg/min).

torr = mmHg, to convert to kPa, multiply the value by 1.333

The main difference between these scores is the definition of cardiovascular failure and that the SOFA score takes into account respiratory support in assessing respiratory failure and oliguria in assessing acute kidney injury (AKI).

Both of these scores, developed to predict morbidity rather than only mortality, enable daily assessment of the organ failures unlike the previously developed scoring systems, such as Acute Physiology and Chronic Health Evaluation (APACHE II) and Simplified Acute Physiology Score (SAPS), which calculate prediction based on assessment on the first 24 hours at the ICU. APACHE score was developed by Knaus *et al.* 1981 and simplified later by the same authors to APACHE II score (Knaus *et al.* 1981, Knaus *et al.* 1985). APACHE II is based on scores of 12 physiological parameters in addition to evaluation of chronic health state. Also, a more precise APACHE III has been

developed, but in its complexity it does not apply to daily usage in all ICU patients (Knaus *et al.* 1991). SAPS scoring is a further simplified model than APACHE that takes into account 13 acute physiological parameters in addition to age (Le Gall *et al.* 1993).

Septic shock is a subset of severe sepsis and is defined as hypotension unexplained by other causes requiring vasopressor treatment. Hypotension is defined by systolic arterial blood pressure of < 90mmHg or mean arterial blood pressure of < 60mmHg or by reduction of systolic blood pressure of 40mmHg despite adequate volume resuscitation. In the presence of perfusion abnormalities like oliguria, lactic asidosis or acute alteration in mental status, the patient is still considered to have septic shock, eventhough inotropic and vasopressor agents would have normalized the blood pressure (Bone *et al.* 1992, Levy *et al.* 2003).

Severe sepsis can be further classified according to the severity of organ failures to multiple organ dysfunction syndrome and multiple organ failure. Multiple organ dysfunction syndrome (MODS) is defined as daily SOFA scores of 1–2 in two or more organ systems on one or more days. The definition of multiple organ failure (MOF) comprises daily SOFA scores 3–4 in two or more organ systems one or more days. (Levy *et al.* 2003)

The most recent staging model for risk stratification in severe sepsis is the PIRO (Predisposition, insult/infection, response and organ dysfunction). It was introduced as a theoretical concept derived from TNM system in oncology (Levy *et al.* 2003) and was further developed and validated against large severe sepsis databases (Rubulotta *et al.* 2009). The first version is not superior to the preceding systems in predicting mortality, but it is less laborious with the potential ability to discriminate morbidity arising from the differentially predisposing factors: infectious insult, host response and organ dysfunction.

2.3 Pathophysiological mechanisms of organ failures in sepsis

Sepsis challenges the homeostatic regulation of the whole body. Initially it was thought that organ dysfunction in sepsis is caused by an excessive inflammatory burst and consequent tissue damage. Now it is commonly understood that genetic features of both pathogen and host as well as hormonal, immune, metabolic and bioenergetic pathways are involved in the development of organ failures (Abraham & Singer 2007). The following chapters summarize the most important ones of the several themes studied in the field of sepsis patophysiology.

2.3.1 Inflammatory response

In sepsis the innate and acquired immunity systems are activated at the whole-body level in order to defend against microbial invasion and restore homeostasis. The inflammatory response is thus crucial to survival, but when dysregulated it can turn against the host leading to organ failure and death (Figure 6). In sepsis pathogenesis a model of proinflammatory response (SIRS, systemic inflammatory response syndrome) followed by a state of immunoparalysis caused by anti-inflammatory response (CARS compensatory anti-inflammatory response syndrome) is supposed (Osuchowski *et al.* 2006). A plethora of key enzymes and mediator pathways have been recognised in immune dysregulation that leads to organ dysfunction, but the system being complex and intertwined, the exact interactions and causality patterns remain unintegrated. How the inflammatory response leads to organ dysfunction is a question, in which no consensus exists as yet.

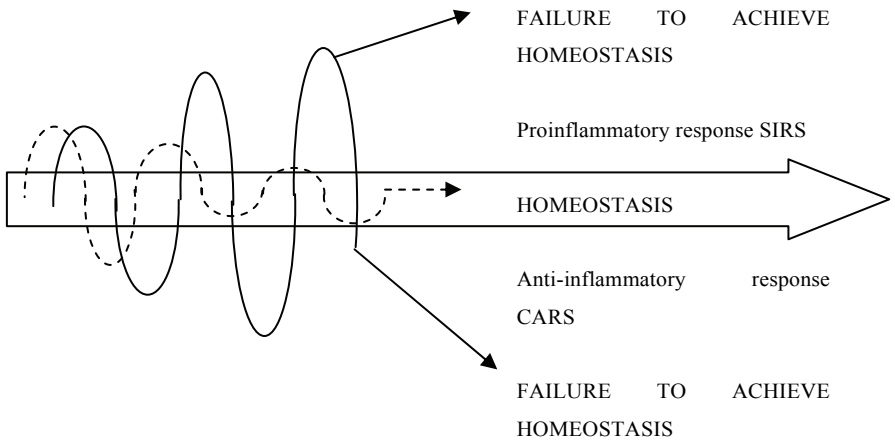

Fig. 6. Sepsis can be seen as a deviation from the normal homeostasis, where several regulative functions are challenged to seek again the balanced state. Inflammatory response in sepsis is characterized by overt inflammation followed by compensatory responses. This is a phenomenon typical to the several physiological feed-back functions maintaining homeostasis in health and disease. If the challenge exceeds the reparative capacities, "oscillation increases" and multiorgan dysfunction and death will follow.

Epithelial barrier dysfunction

The body is divided into distinct compositionally and functionally different units by epithelial sheets lining every organ. An essential element of the formation of epithelial sheet, are the tight junctions between the epithelial cells. They form a semipermeable barrier that, in addition to transcellular routes for molecular change, regulates the exchange of molecules and solutes and generates distinct internal environments vital to appropriate functioning. In organs lining the body surfaces such as the lung and gut, this barrier function serves also as a defence against invasion by microbes and toxins from the external environment. In addition, epithels secrete antimicrobial peptides and chemokines. Epithelial barrier dysfunction is one of the major features in the pathofysiology of multiple organ failure and severe sepsis (Fink & Delude 2005). Increased intestinal permeability is associated with the development of organ dysfunctions in critically ill (Doig *et al.* 1998). Histopathological evidence of decreased epithelial tight junction protein expression has been demonstrated in the ileum, liver, lung and endothelium of endotoxemic mice (Fink & Delude 2005, Maas *et al.* 2005) and in the gallbladder epithelium (Laurila *et al.* 2007) of critically ill patients.

With electron microscopy it was seen that labelling of claudins was present diffusely in the intestinal epithelial cell and not on lateral boundaries. It was also observed that this disruption of tight junction structure was accompanied by increased intestinal permeability (Qiurong *et al.* 2009). Epithelial dysfunction in the gut is suggested to be mediated by TNF-α and high mobility group box 1 (HMGB 1) (Yang *et al.* 2009). Recently it has been also shown that thrombin and other PAR-1 agonists disassemble claudin-5 from tight junctions of endothelial cells and in this way increase vascular permeability (Kondo *et al.* 2009).

Triggering the host response

Sepsis is caused by an excessive host response to the invading micro-organism and their products. The pathogen specific structures the innate immunity is able to detect are called pathogen-associated molecular patterns (PAMPs), for instance the gram-negative bacterial cell wall component lipopolysaccharide (LPS). LPS is released upon bacterial lysis with endotoxic complex forming a proteinaceous solute called endotoxin. PAMPs also include lipoteichoic acid of gram-positive bacteria, peptidoglycan in gram-positive and negative bacteria, flagellin in flagellated bacteria, viral RNA and mannan in candida albicans Alarmins are a

molecule group released in tissue damage, that innate immunity additively recognises, and together with PAMPs form a group of Damage associated molecular patterns (DAMPs) (Bianchi 2007).

The innate immunity – the first line defence

The innate immunity response enables rapid recognition and removal of invading pathogens and primes the adaptive immunity response. The innate immunity consists of the mechanisms briefly introduced in Figure 7.

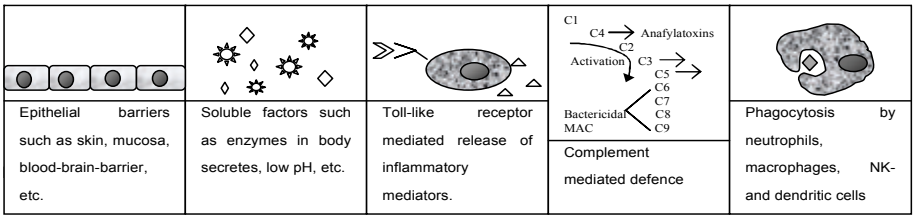

Fig. 7. Mechanisms of innate immunity.

Toll-like receptors

The discovery of toll-like receptors (TLR), transmembrane proteins on immune cells, has increased our understanding of early pathogen recognition and activation of inflammatory cascades. The surface receptor of TLR senses conserved soluble antigens that the pathogens release and this activates the intracellular TIR-domain. A downstream kinase cascade leads to phosphorylation and dissociation of inhibitor kappa B-cell from the nuclear factor kappa B (NF-κB). NF-κB thus becomes able to translocate to the nucleus and activate the transcription of inflammatory cytokines, chemokines, acute phase proteins and other inflammatory mediators. (Opal & Cristofaro 2007)

Cells of the innate immunity

Neutrophils are suited for rapid elimination of pathogens due to their capacity to produce reactive oxygen species and proteolytic enzymes that degrade phagocyted pathogens. The response is unspecific and tissue damage will occur where neutrophils are activated. Systemic neutrophil activation is a central feature

of sepsis pathofysiology. However, in severe sepsis the neutrophil recruitment is supposed to be defective, with inappropriate high infiltration of these cells to the lung tissue (Kinoshita *et al.* 1999) and migration failure with aggregates in blood vessels in other organs such as the brain (Zhou *et al.* 2009) and skin (McGill *et al.* 1996). The mechanisms by which neutrophils are suggested to participate in causing organ damage are hypoxia caused by neutrophils occluding microcirculation and persistent overt inflammatory activity in tissues. The latter is partially caused by neutrophil apoptosis inhibition observed in experimental sepsis models (Jimenez *et al.* 1997, Taneja *et al.* 2004)

Cells of the monocyte/macrophage lineage are multifunctional cells that play a central role in the pathogenesis of severe sepsis and related organ failures. The functions of the macrophages upon inflammation are summarized in Figure 8. The activation profile is different depending on the tissue type and the biochemical milieu created by cytokines and growth factors.

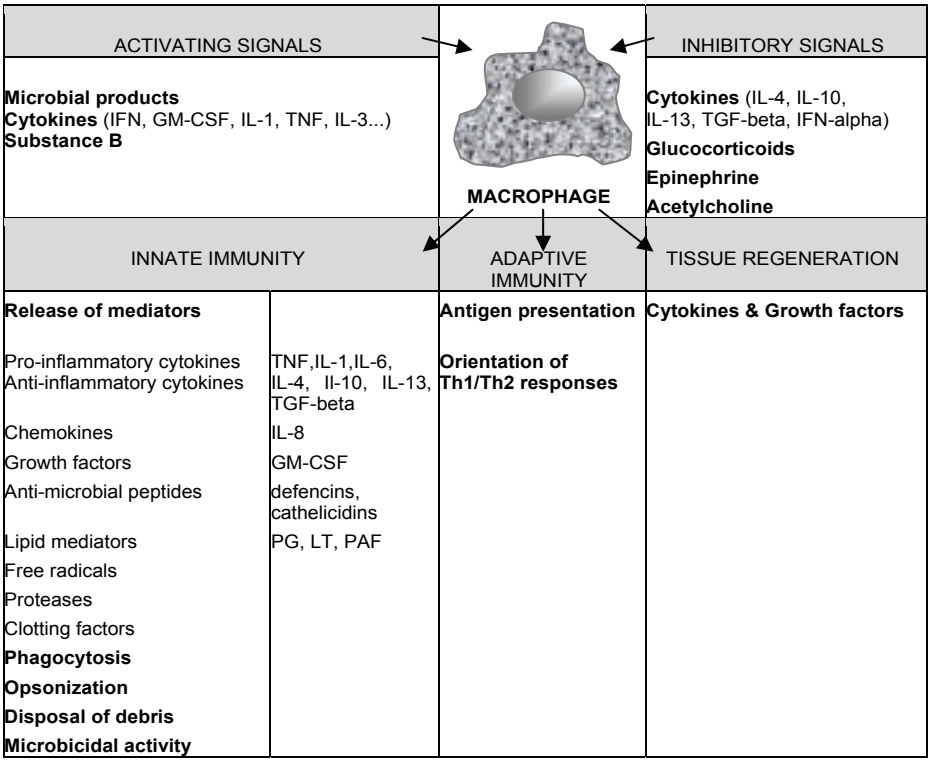

Fig. 8. Activating and Inhibiting signals and functions of monocytes/macrophages in inflammation (modulated from (Cavaillon & Adib-Conquy 2005) with permission).

Cytokines

Cytokines are a family of protein mediators essential to interactions between cells of inflammatory response. They are typically released from immune cells in response to inflammatory stimuli. It is typical for cytokines that one cytokine can stimulate several cell types (pleiotropism), and that different cytokines can have similar effects (redundancy). Cytokines also regulate the secretion of other cytokines. The cytokines are divided into pro-inflammatory and anti-inflammatory cytokines according to the activities they induce. However, several cytokines are shown to exert both roles depending on the biochemical milieu. This and evolution of inflammatory signals in time and the complex interactions are thought to contribute to the fact that several clinical trials attempting to modulate the cytokine profile in order to impact the survival rate have failed. Tumor necrosis factor is the most profoundly studied pro-inflammatory cytokine in systemic inflammation. It is produced by various inflammatory cells and it exerts effects by binding to TNFα type I or type II receptors. TNFα activates myeloid cells and triggers the release of multiple inflammatory mediators (cytokines including also TNFα, nitric oxide (NO), platelet activating factor (PAF), prostaglandins and free radicals). Among the esulting effects there are hepatic synthesis of acute phase proteins, shock, capillary leak and myocardial depression and chemotaxis and recruitment of inflammatory cells. Since TNFα infusion was shown to cause symptoms of sepsis in animals, the pivotal role of this cytokine in sepsis pathogenesis has been acknowledged in many experimental and clinical studies (Kaech & Calandra 2007). The downstream effects of TNFα on tissue regeneration and fibrotic processes have also been addressed in various studies (Kovacs & DiPietro 1994). Han et al reported that TNFα, in a collagen rich environment, activates MMP-2 and proposed that this is mediated via intracellular release of NF-κB, resulting in the increase in Membrane type matrix metalloproteinase-1 (MT1-MMP) expression and MT1-MMP mediated MMP-2 activation (Han *et al.* 2001b). The same group demonstrated also that expression of pro-MMP-9 and cleavage to active enzyme is induced by TNFα as well as TGFβ (Han *et al.* 2001a). Other common cytokines and their cellular sources and actions are presented in Table 7.

Table 7. The central cytokines in severe sepsis and their functions. (Adapted by permission from (Karlsson 2009)).

Cytokine	Cellular sources	Major activities
TNF-α	Monocytes	Proinflammatory
	Macrophages	promotes inflammation
		activates macrophages and neutrophils
		induces the production of adhesion molecules in endothelial cells
		induces NO synthase in endothelial cells
		activates complement system
		activates coagulation
IL-1β	Monocytes	Proinflammatory
	Macrophages	promotes inflammation
		activates macrophages and T cells
		potentiates the effects of TNF-α
IL-1ra	Monocytes	Anti-inflammatory
	Macrophages	IL-1-receptor antagonist (ra)
	Dendritic cells	inhibits IL-1 –mediated cellular activation
IL-2	T helper cells	Proinflammatory
	(Th1)	activates lymphocytes, natural killer cells and macrophages
IL-4 T helper	Mast cells	Anti-inflammatory
cells (Th2)	B cells	inhibits LPS-induced proinflammatory cytokine synthesis
	Stromal cells	promotes Th2 lymphocyte development
IL-6	Macrophages	Proinflammatory
Monocytes	Endothelial cells	activates lymphocytes
	Polymorphonucle	differentiation of B cells
	ar	stimulates the production of acute phase proteins
	cells	Anti-inflammatory
		inhibits TNF and IL-1 production by macrophages
		stimulates adrenocorticotrophic hormone
IL-8	Macrophages	Proinflammatory
Monocytes	Endothelial cells	chemotaxis of neutrophils, basophils and T-cells
IL-10	T cells (Th2)	Anti-inflammatory
	B cells	inhibits monocyte, macrophage and neutrophil cytokine production
	Monocytes	inhibits Th1 lymphocyte responses
	Macrophages	inhibits IL-2 and IFN-γ
		inhibits NF-κB nuclear translocation
L-11	Fibroblasts	Anti-inflammatory
	Bone marrow	inhibits monocyte/macrophage proinflammatory cytokine response
	stromal cells	promotes Th2 lymphocyte response
IL-13	T cells (Th2)	Anti-inflammatory
		like IL-4
		attenuates monocyte and macrophage function

Cytokine	Cellular sources	Major activities
Interferon-γ	T cells (Th1)	Proinflammatory
(IFN-γ)	NK cells	activates macrophages
		inhibits Th2 lymphocyte responses
HMGB1	Macrophages	Proinflammatory
	Dendritic cells	stimulates monocytes to produce TNF-α, IL-1, IL-6
	Natural killer cells	regulates fibrinolysis by secreting PAI-1 and tPA
	Necrotic cells	
Macrophage	Monocytes	Proinflammatory
migration	Macrophages	activates T cells
inhibitory	T cells, B cells	stimulates macrophages
factor (MIF)	Epithelial cells	modulates the expression of TLR4 on macrophages

Acquired immune response

The antigen specific B- and T-lymphocytes are the central effector cells of acquired immunity. As a response to contact with pathogenic structures B-cells proliferate and produce antibodies. The T-cells maturate in the thymus to CD8+ cytotoxic T-lymphocytes that recognise pathogens presented by major histocompability (MHC) class I molecules, or to CD4+ helper T-lymphocytes that recognise pathogens presented by MHC II class molecules. The cytokine profile CD4+ cells produce divides them into the groups of Th1 and Th2 cells. Th1 cell mediated functions are typically pro-inflammatory while Th2 mediates anti-inflammatory responses. Sandler *et al.* found that Th2 polarized mice upregulated genes associated with MMPs, collagens and wound repair while Th1 polarized mice upregulated genes associated with tissue damage (Sandler *et al.* 2003).

Resolution of the inflammation

The resolution of inflammation requires that the pathogen initiating inflammatory signal is removed, the cytokine cascades dissipated and the infiltrating immune cells removed from the tissues. Ultimately the tissue returns to normal function and structure, but when inflammation remains persistent it can lead to ongoing fibroproliferation and compromises the normal functions. Interestingly in sepsis most organs seem to recover completely after severe functional changes.

Anti-inflammatory cytokines, like interleukin receptor antagonist (IL-1ra), IL-4, IL-10 and IL-13, act to limit the inflammation and promote resolution. Also

non-signalling forms of cytokine receptors attenuate the signals by binding excessive pro-inflammatory cytokine.

Infiltrated immune cells are removed through apoptosis or emigration to the lymphatic tissue. Apoptosis is a natural fate of leukocytes. In sepsis the inflammatory mediators delay neutrophil apoptosis in circulating neutrophils (Jimenez *et al.* 1997, Taneja *et al.* 2004). But after the inflammation resolves neutrophils undergo the controlled cell death and are removed by macrophages (Ishii *et al.* 1998). In contrast to neutrophils, macrophages do not seem to be removed by apoptosis, but emigrate to the lymph nodes (Bellingan *et al.* 1996). The resolution of the inflammation seems to be a highly controlled event and the underlying mechanisms represent an interesting field for further investigation.

2.3.2 Microcirculatory dysfunction

All in all, septic circulation is characterized by marked heterogeneity of vascular response not only between organ systems, but also in time. Early sepsis is characterised by vasodilatation, capillary leak and insufficient intravascular volume. Low cardiac output with myocardial depression causes perfusion abnormalities and consequent depletion in oxygen delivery. Early fluid resuscitation improves oxygen delivery and clinical outcome (Rivers *et al.* 2001). But despite the restoration of hemodynamics multiple organ failure develops frequently. Therefore the role of microcirculatory failure and mitochondrial dysfunction has been emphasized recently in the patophysiology of septic organ failures.

The hallmark of microcirculatory studies in sepsis has been the development of orthogonal polarization spectral imagining (OPS). De Backer showed with OPS that functional sublingual vessel density and perfusion in vessels smaller than 20μm were depressed in patients with severe sepsis (De Backer *et al.* 2002). Microvascular shunting, despite sustained flow in larger vessels, seems to be one feature of septic circulation (Ince & Sinaasappel 1999, Spronk *et al.* 2002). The "shutting down" of this crucial exchanger of oxygen, nutritients, waste products and signalling molecules could indeed offer another explanation for organ failures. Disseminated intravascular coagulation occludes the capillaries and consumes proteins (Zeerleder *et al.* 2005). Adhesive and activated platelets and neutrophils and deformed red blood cells make the picture even more blurred (Hinshaw 1996).

In addition endothelial dysfunction is one major pathophysiological component. Capillary wall consists of endothelium surrounded by occasional

pericytes. In sepsis the endothelium participates actively in host defence by producing pro-and anticoagulants, inflammatory mediators and vasomotor agents like NO. The resulting functional changes include turning the hemostatic balance to procoagulative, increased leukocyte adhesion and trafficking, altered vascular tone and increased permeability and increased apoptosis (Figure 9). (Aird 2003)

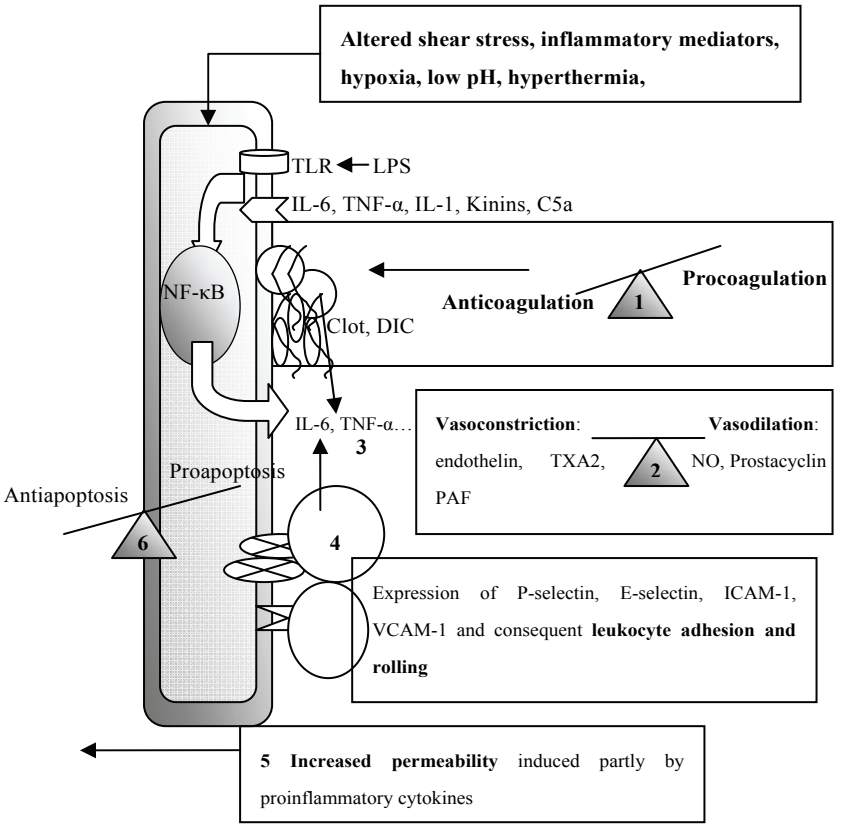

Fig. 9. Simplified presentation of activated endothelial cell and consequent functional changes. After the endothelial cell is activated via toll-like receptors or different receptors of inflammatory mediators (drawn as single representative receptor), NF-κB and other transcriptional factors are released into nucleus. Consequently endothelium activates and primes the coagulation cascade (1), releases vasoactive agents (2), inflammatory mediators (3) and expresses leukocyte adhesion molecules. Additionally, changes in expression of proapoptotic genes increase (6). (Aird 2003). TLR, toll-like receptor, LPS, Lipopolysaccharide, IL, Interleukin, TNF, Tumour necrosis factor, NF-κB, Nuclear factor kappa beta, DIC, Disseminated intravascular coagulation, TXA2, tromboxan A2, PAF, Platelet activating factor, ICAM-1, intracellular adhesion molecule 1, VCAM-1, vascular cell adhesion molecule-1.

2.3.3 Mitochondrial malfunction

Mitochondrial malfunction is a fascinating theory of patofysiology of septic organ failures. The fact that cells would hibernate upon the septic insult to overcome the major homeostatic challenge would explain why the level of organ failure is never in proportion with morphological changes (Hotchkiss *et al.* 1999). Evidence of mitochondrial dysfunction in human sepsis is still scanty. Brealey et al have found association between skeletal muscle mitochondrial function and sepsis severity with 28 patients and the result could be repeated in a following study with rats, both liver and kidney, presenting complex I inhibition and fall in ATP levels in more severely septic animals (Brealey *et al.* 2002, Brealey *et al.* 2004).

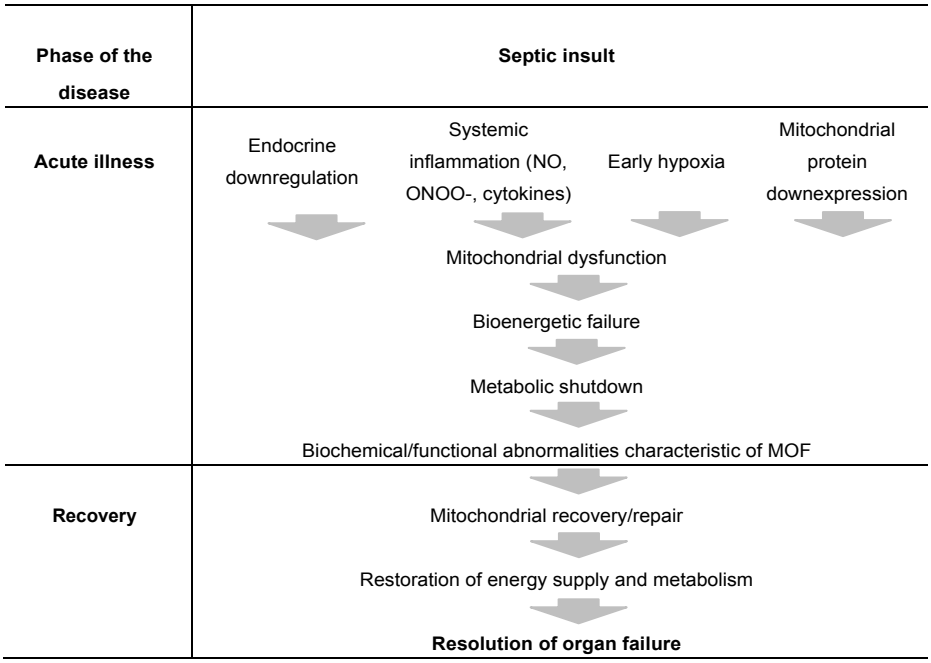

Fig. 10. Hypothesized role of mitochondria in the development of multiple organ failure and subsequent recovery (Modified with permission from (Protti & Singer 2006).

2.4 Connective tissue metabolism in sepsis

Fibrotic response is a vital continuum of the normal healing process ascertaining tissue integrity after injury. However, when excessive it can lead to the loss of organ function. This occurs for instance in ARDS, which is common organ failure in severe sepsis. Fibroproliferation occurs earlier than previously recognized, already from the first day on, and impacts outcome (Marshall *et al.* 2000b). Waydhas *et al.* were first to report elevated PIIINP levels in patients with MODS and MOF and its association with poor outcome (Waydhas *et al.* 1993). Pathological fibrosis is a common feature in prolonged inflammation, but in sepsis the role of fibroproliferative response in the development of organ dysfunction is poorly studied. Fibrosis is one potential patophysiological mechanism of organ dysfunction in sepsis. Furthermore as our knowledge on the patophysiological changes in tissue healing in sepsis increases, we may obtain a better understanding of how to manage tissue healing problems which frequently arise in critically ill sepsis patients.

2.4.1 Fibroproliferation as a continuum of healing response

The extracellular matrix is constantly remodelled by equilibrium of matrix protein synthesis and degradation. Fibroblasts and myofibroblasts are the cells mainly responsible for extracellular matrix turnover by producing growth factors and cytokines and secreting extracellular matrix proteins (Kisseleva & Brenner 2008b). In injury tissue fibroblasts respond to a plethora of mechanical and chemical stimuli arising from coagulation and inflammation cascades summarized in figure and discussed in following chapters (Figure 11).

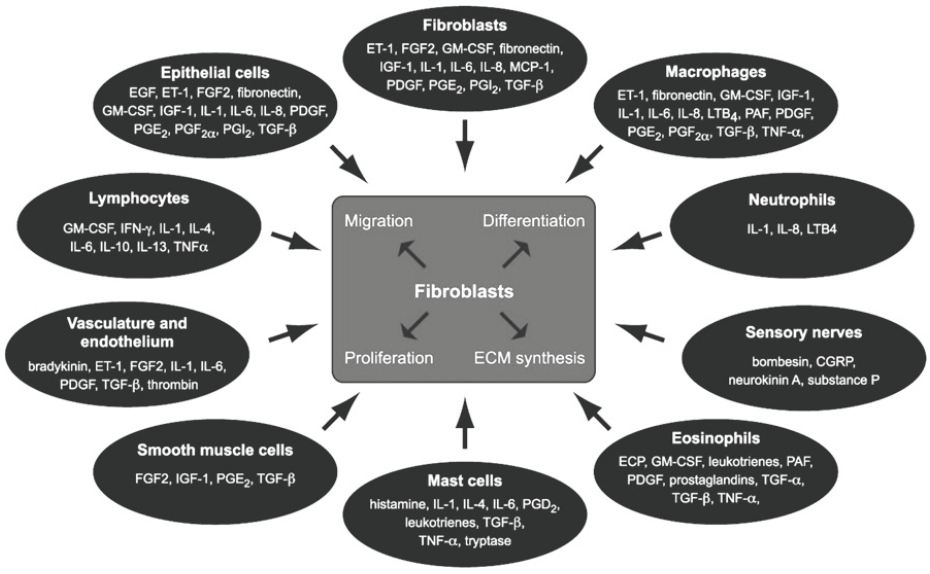

Fig. 11. Factors regulating fibroblast function. CGRP = calcitoningene-related peptide; ECP = eosinophil chemotacticprotein; EGF = epidermal growth factor; ET-1 = endothelin-1; FGF2 = fibroblast growth factor 2;GM-CSF = granulocyte-macrophagecolony–stimulatingfactor; IGF-1 = Insulin-like growth factor-1; LTB4 = leukotriene B4; MCP-1 = monocyte chemotacticprotein-1; PAF = platelet activating factor; PDGF = platelet-derived growth factor; PGD2 = prostaglandinD2; PGE2 = prostaglandin E2; PGF2a = prostaglandinF2a; PGI2 = prostacyclin; TGF-α = transforming growth factor- α; TGF-β = transforming growthfactor- β; TNF- α = tumor necrosis factor- α. Reprinted from (McAnulty & Laurent 2002) by permission from Elsevier.

Activated fibroblasts begin proliferating, migrating and differentiating to myofibroblasts. In addition to tissue fibroblasts, myofibroblasts can arise from hepatic stellate cells, bone marrow derived fibroblasts and fibrocytes or transformate from hepatocytes, monocytes, epithelial or endothelial cells (Haudek *et al.* 2006, Kisseleva & Brenner 2008b, Zeisberg *et al.* 2007). Evidence of this plasticity between parenchymal and fibrogenetic cells interestingly links the studies of apoptosis control and fibrotic response. Myofibroblasts are characterized by expression of α-smooth muscle cell actin and myosin that enable contractile properties (Walker *et al.* 2001). In acute wound this deposition of extracellular matrix proteins follows a certain pattern with fibronectin and hyaluronic acid production followed by collagen type III and then type I production (Kurkinen *et al.* 1980). Simultaneously to matrix production, a

constant remodellation and organisation takes place. This is a process where the balance of matrix-metalloproteinases and their inhibitors play a central role. It is well known that chronic inflammation leads to progressive fibrosis and organ failure (Weber 1997, Kisseleva & Brenner 2008a). However, clinical experience of acute organ failures indicates that the resolution of healing response can be adaptive and can lead to restoration of organ function. ARDS is the most studied acute organ failure in this perspective. Yet factors influencing progression to fibroproliferative ARDS versus resolution of fibrosis and retaining normal architecture and function are poorly understood. (Figure 12)

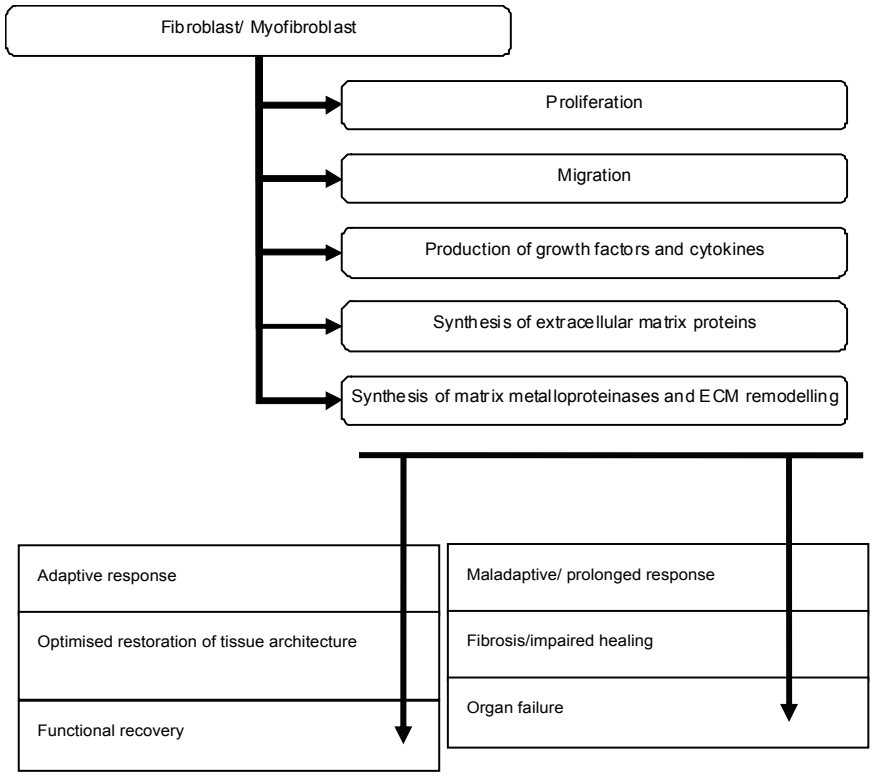

Fig. 12. Functions of activated fibroblasts and pathways of outcome in fibrosis.

The healing response is thought to follow a similar pattern regardless of the tissue involved. In summary, coagulation and inflammation are followed by granulation tissue formation. This provisional matrix develops further by proliferation and

differentiation of cells to regenerated functional tissue and neovasculature with gaps filled with collagenous scar tissue. In sepsis the healing response is featured by activated coagulation and inflammation involving the whole body. To which extent the fibroblasts are activated in organs affected by sepsis, is a question of arising interest.

2.4.2 Activated coagulation as regulator of fibroblast response

Patients with severe sepsis have almost invariably activated coagulation. Activation of tissue factor dependent coagulation and concurrent impairment of anticoagulative functions characterise the disseminated intravascular coagulation in severe sepsis (Zeerleder *et al.* 2005). On the other hand, procoagulative proteases are known to exert regulation to fibrotic response. Activated coagulation protease X seems to increase the expression of the main fibrogenetic cytokine TGF-β, fibroblast proliferation and differentiation to myofibroblasts, fibroblast migration and fibronectin production (Borensztajn *et al.* 2008). Also, thrombin seems to be profibrotic by being fibroblast chemoattractant (Dawes *et al.* 1993), stimulator of procollagen production (Chambers *et al.* 1998), promoter of myofibroblast formator (Bogatkevich *et al.* 2001) and MMP activator (Duhamel-Clerin *et al.* 1997). Thrombin and Xa exert their effects via proteinase activated transmembrane receptors (PARs). PAR-1, which is activated both by thrombin, factor Xa and even plasmin, is shown to be important in the pathogenesis of acute lung injury. Using bleomycin induced lung injury as a model of ALI/ARDS Howell *et al.* demonstrated that thrombin and PAR-1 are central in mediating inflammation, microvascular leak and fibrotic response in ARDS. Bleomycin injury increased thrombin and PAR immunoreactivity in lung. In addition thrombin antagonist significantly attenuated lung procollagen gene expression and collagen accumulation following injury (Howell *et al.* 2001). PAR -/- knockout mice presented with dramatically reduced collagen accumulation, inflammatory cell count and microvascular permeability compared with wild-type mice after bleomycin injury (Howell *et al.* 2005).

2.4.3 Regulative role of inflammatory cascade in fibrogenesis

The number of potential inflammatory mediators affecting fibrotic response in acute organ failures in severe sepsis is huge (Figure 11). Although experimental models and studies on chronic fibroproliferative diseases offer evidence of the

roles of pro-and anti-fibrotic mediators, little is known about those that directly regulate fibrogenesis and its resolution in ARDS or other organ failures in severe sepsis. TNF-α is a central inflammatory mediator in sepsis and regulates fibroplasia and collagen synthesis in multiple ways. In addition to stimulating fibroblast growth and collagen synthesis, TNF- α has been shown that in high concentrations it inhibits collagen and fibronectin production and induces synthesis of collagenases (Maish *et al.* 1998). Among growth factors TGF-β is essential. High levels of TGF-β have been reported in trauma patients developing sepsis (Laun *et al.* 2003). In ARDS it has been demonstrated that bronchoalveolar lavage fluid obtained from patients activates procollagen I promoter by means of TGF-β. Furthermore, TGF-β levels are slightly higher in non-surviving ARDS patients (Budinger *et al.* 2005).

2.4.4 Other sepsis associated factors affecting fibroblast function

Other sepsis associated factors that have been shown to affect fibroblast function include mechanical loading, ischaemia-reperfusion injury and apoptosis. Experimental studies have revealed that mechanical stretch induces expression of type I and II collagens and cyclic loading induces expression of MMP-2 from rat cardiac fibroblasts. In addition, hypoxia induces differentiation to myofibroblasts, reduces cardiofibroblast proliferation, and invasion induces collagen synthesis and may induce MMP-2 expression. Also, reoxygenation is a potent enhancer of collagen, MMP and TIMP synthesis in cardiac fibroblasts (Porter & Turner 2009).There are also interesting links between apoptosis of parenchymal cells and fibroproliferation. Fas-ligand is a membrane protein on inflammatory cells and is cleaved to soluble form by MMPs. Membrane bound and soluble Fas-ligands bind to Fas (apoptotic signal initiating membrane receptor) which triggers caspase mediated apoptotic pathway. In mice with inactive Fas, bleomycin induced lung fibrosis was markedly decreased (Hagimoto *et al.* 2002). Thus, inflammation promotes apoptosis and apoptosis seems to promote fibrosis, which seems reasonable as the aim of the fibrotic response is to fill the gaps resultant from parenchymal cell death.

2.4.5 Connective tissue metabolism at organ level in sepsis

Disordered connective tissue metabolism is evident in lung involvement in severe sepsis (ARDS). In other *acute* organ dysfunctions evidence of fibrosis is more

scant. The following chapters discuss organ by organ the role of fibrosis in acute organ dysfunction, the mechanisms driving the fibrotic response, and what is known of the factors terminating the fibrotic response and leading to adequate healing of the tissue structure.

Lung

Acute lung injury (ALI) and its most severe manifestation acute respiratory distress syndrome (ARDS) characterize the involvement of the lung in severe sepsis. ARDS with sepsis etiology has higher mortality than in trauma or other non-infectious etiology (60%, 43% and 10–22%, respectively) (Monchi *et al.* 1998). Two overlapping phases are recognized in the pathogenesis of ARDS:

1. *Exudative phase* is characterized by increased alveolar barrier permeability and resultant inflow of cells and proteinaceous exudate into alveolar space. This is followed by type I and II pneumocyte cell death, endothelial damage and surfactant denaturation.
2. *Proliferative phase* in which pneumocyte, fibro- and myofibroblast proliferation and extracellular matrix deposition take place.

Of ventilated ARDS patients 53% present with fibrosis in open lung biopsy specimens (Papazian *et al.* 2007). Fibrosis begins already on the first day of the disease and is associated with mortality when compared with patients without fibrosis (Marshall *et al.* 2000b, Santos *et al.* 2006).

Factors central to the development of fibroproliferative ARDS are persistent inflammation, disordered matrix remodellation and inadequate cellular apoptosis and proliferation (Rocco *et al.* 2009). Among the various cell signalling processes ongoing in inflammation and repair, signalling between activated macrophages and fibroblasts is considered to be central to initiation and progression of fibrosis. Macrophages are the main resources of profibrotic cytokines and growth factors such as TNF-α, IL-1, TGF-β (Cavaillon & Adib-Conquy 2005). BAL fluid from ARDS patients has been shown to contain TGF-β, which activated procollagen I promoter in vitro (Budinger *et al.* 2005). Removal of ongoing TGF-β signalling is associated with resolution of fibrotic response in the lung. Besides the cytokines and cell-cell interactions, the active role of ECM components in regulating fibrosis has recently been acknowledged. Collagen, matrix metalloproteinases and other ECM components extend regulative effects through cell-ECM interactions

(Rocco *et al.* 2009). Expression of matrix metalloproteinases and their inhibitors has been shown to be altered in severe sepsis (Hoffmann *et al.* 2006). Also, the evidence of the role of dysregulated apoptosis is increasing. Finally, it is well documented that mechanical ventilation promotes fibrotic response. The use of steroids is not unproblematic as antifibrotic therapy in ARDS. Promising results on new antifibrotic therapies with angiotensin II inbibitors and stem-cell therapy are accumulating (Rocco *et al.* 2009).

Skin

Only a few studies address the effect of systemic inflammation to skin fibrogenetic response and cutaneous wound healing in sepsis. Yet the skin being the largest organ and the main defensive barrier, the issue of skin failure should be addressed in the context of septic organ failures. This issue is addressed in chapter 2.5.

Liver

Little is known about the histopathology of acute liver failure in severe sepsis, whereas the process of fibrosis in chronic liver failure is studied in detail. In septic liver failure apoptosis in both inflammatory cells and liver cells are seen (Hotchkiss *et al.* 1999), but evidence of fibrotic activity is lacking. As liver fibrosis is the final common pathway in almost all chronic liver diseases and as it is now known that resolution of liver fibrosis to near to normal tissue architecture can occur (Iredale 2007) it is temptating to speculate that ECM remodelling also takes place in liver during sepsis. TGF-β released by macrophages in sepsis promotes transition of hepatic stellate cells to myofibroblast-like phenotype and collagen I expression. As mentioned above, the prevention of apoptotic signalling ameliorates lung fibrosis (Hagimoto *et al.* 2002), thus providing evidence on regulative links between cell death and ECM remodelling. In liver, apoptosis of hepatocytes is suggested to activate hepatic stellate cells to myofibroblast-like collagen producing cells indirectly via ROS and cytokine production (Kisseleva & Brenner 2006). This phenomen has not yet been studied in septic liver.

Kidney

As with other organs the pathology of acute kidney injury (AKI) is multifactorial. Previously hemodynamic alterations have been highlighted, but as renal failure occurs despite resuscitation of global hemodynamics other factors seem to be involved. Interesting hypothesis generating insights into the role of fibrosis in AKI can be derived from an experimental study with AKI in rats. Ischemic injury resulted in hypertrophy, which was due at least in part to interstitial cells that stained positively for a fibroblast specific marker. TGF–β is known to induce fibrosis and anti TGF-β antibody attenuated renal hypertrophy. Furthermore, TGF-β neutralisation also attenuated the loss of renal vascular density following ischemia-reperfusion injury (Spurgeon *et al.* 2005). Keller *et al.* noted in their study on hemodialysis patients that PIIINP levels were 3-fold higher in patients with chronic kidney failure in comparison with controls, but five patients with AKI as a manifestation of MOF had 10-fold higher levels. Hemodialysis had no effect on the elimination of the PIIINP (Keller *et al.* 1988). Further evidence of fibrogenetic mechanisms in the repair of AKI can be obtained from a research by Fujigaki *et al.* (Fujigaki *et al.* 2005) After induction of AKI, peritubular α-SMA positive myofibroblasts appeared and extended along the damaged proximal tubules and almost disappeared after recovery. Inhibiting cytoskeletal movement and myofibroblast differentiation resulted in more dilated proximal tubules, more severe renal dysfunction and inhibition of regenerative repair. The authors conclude that the mechanical tension from dilating tubules might induce the α-SMA phenotype and that the increased tension fibre formation and intercellular junctions serve to support damaged nephron structures during repair.

Heart and circulation

Studies on alterations on extracellular matrix in cardiac tissue in acute heart failure in sepsis are lacking. However excessive evidence on myocardial remodelling due to ischaemia and hypertension include mechanisms that may analogiously function in septic heart failure. Cardiac fibroblasts are known to respond to mechanical loading, ischaemia reperfusion injury, neurohormonal stimuli and some of the key cytokines known to be elevated in septic circulation. For instance, the key cytokine in sepsis, TNF-α increases migration, proliferation, expression of MMPs and expression of proinflammatory cytokines in human atrial fibroblasts (Porter & Turner 2009). Epstein *et al.* investigated intimal

remodelling after ballon injury to iliac artery in the presence of septic insult. When high dose of LPS was given 72h prior injury, the neointimal formation was decreased, whereas high dose immediately after injury did not affect neointima formation while low dose immediately after injury increased neointima formation (Epstein *et al.* 2008).

Gastrointestinal tract

In rats, sepsis has been shown to impair the healing of colonic anastomosis, which was associated with decreased collagen concentration (Ahrendt *et al.* 1994). In another rat colonic anastomosis study it was shown that in endotoxemia, tissue from septic rats had less capacity to synthesize collagen than tissue from control rats (Thornton *et al.* 1997). Both type I and III collagen mRNA expression was decreased in endotoxemia.

2.5 The skin and sepsis

The concept of skin failure as a part of multiple organ failure was introduced already in 1991 and the term was further defined by Langemo and collegues (Langemo & Brown 2006) simply by description: "an event in which the skin and underlying tissue die due to hypoperfusion that occurs concurrent with severe dysfunction or failure of other organ systems". Yet it is the other body lining organs such as the intestines and lungs that have been in the focus in severe sepsis studies. However, the skin can fail like other organs and one evidence of this is the high prevalence and incidence of pressure ulcers in intensive care settings. According to a review by Van Gilder et al., the point prevalence of pressure ulcers in hospitalized population is 14%, whereas in intensive care setting the prevalence ranges between 22.4–25.9% (Vangilder *et al.* 2008). According to a systematic review from year 2000 to 2005 the prevalence ranged from 4% to 49% and incidence from 3.8% to 12.4 % (Shahin *et al.* 2008). Pressure ulcer occurence is also known to be associated with multiple organ failure, high APACHE-score and infection (Compton *et al.* 2008, Eachempati *et al.* 2001). The pathophysiological studies on pressure ulcers have been concentrated, as the name also implies, on external factors. However, it is evident that possible intracutaneuous pathology also increases the susceptility to damage caused by external forces. Deeper understanding of this internal patophysiology will in future help to detect danger before damage and create ways to interfere early enough.

2.5.1 The structure and function of the skin

The skin compromises approximately 10% of body weight. It is structurally divided into epidermis and dermis that are divided by the basement membrane (Figure 13). Epidermis is divided into zones according to the differentiation grades of the keratinocytes: the basal, spinous, granulous, and horny layers.

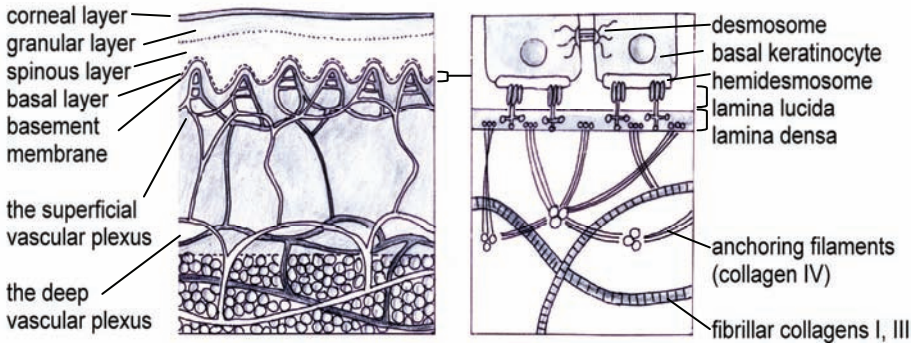

Fig. 13. Structure of the skin with vasculature and basement membrane zone in focus.

The epidermis is a stratified squamous epithelium of keratinocyte cells. It is constantly regenerating from the one cell layer thick basal layer. As the basal keratinocytes divide, the surplus of cells move onwards and differentiate to prickle cells in the spinous layer that is 5–10 cell layers thick. Spinous cells are rich in keratinfibrils. The keratinocytes are connected to each other with desmosomes, and by constant de- end reattachment they move towards the surface of the skin. In the granulous layer the cells are filled with keratin and keratinhyalin. Lastly, as the cells became flattened and the nuclei disappear, the horny layer (stratum corneum *la)* is formed. In this uppermost layer the keratinocytes are surrounded by matrix rich in lipids.

The dermis is mainly composed of acellular matrix with collagens I and III building up 70% of the dry substance. 85% of the collagen is of type I and 15% of type III, additively the basement membrane zone contains collagens IV and VII. The network of elastin gives the skin its elastic properties. Glykosaminoglycans bind water and adhere to proteins thus forming proteoglycans. The main cell producing these extracellular matrix components is fibroblast. In addition, the dermis contains few lymphocytes and mastcells (Oikarinen & Tasanen-Määttä 2003). The functions of the skin are outlined in Table 8.

Table 8. Functional properties of the skin (modified from (Oikarinen & Tasanen-Määttä 2003)).

Function	Properties of the skin enabling functionality
Protection against	
Micro-organisms	Lipids and peptides of stratum corneum; Cells and their products in the epidermis; Antigen presenting Langerhans cells, Cytokine and growth factor producing keratinocytes; mast cells containing cytokines and enzymes and lymphocytes producing cytokines and antibodies
Mechanical forces	Elasticity and strength giving elastins, collagens and other dermis components and the desmosomes and hemidesmosomes of the epidermal keratinocytes.
UV-radiation	Pigment producing melanocytes of the epidermis, stratum corneum
Chemical irritation	Stratum corneum, Epidermis
Water loss	Stratum corneum, Epidermis. Tight junctions of the epidermal keratinosytes.
Metabolism of vitamin D	Epidermis
Temperature regulation	Sweat glands/Blood circulation
Sensing of tactile and thermal stimulus	Free nerve endings, Pacini corpuscles, Meissner´s corpuscles

2.5.2 Skin barrier function

The skin maintains homeostasis by being a barrier against external forces, chemicals and pathogens and by regulating loss of fluids. The importance of this barrier is highlighted in conditions where it is lost. In pemfigus vulgaris, toxic epidermal necrolysis and severe burns, the epidermis becomes loose due to deep blistering. When extensive, these conditions are life threatening and patients die because of extensive water loss and microbial invasion that induce systemic inflammation. (Proksch *et al.* 2008)

The stratum corneum contributes to barrier function with the protein-enriched flattened denucleated keratinocytes in the matrix of hydrophobic lipids (a part of which are direct antimicrobial) (Elias 2005).

Also, the nucleated epidermis below stratum corneum is significant for the barrier function. Tight junctions seem to take part in restricting fluid permeability. Knock-out mice of tight junction proteins present excessive water loss. (Proksch E. *et al.* 2008) In skin injuries, tight junction protein expression is upregulated early before stratum corneum formation (Malminen *et al.* 2003), and thus it can be speculated that the formation of tight junctions serves as prelimary defence against water loss during wound healing.

The microbial invasion is hampered in addition to the physical barrier by innate immunity of the skin. Mechanisms of innate immunity in skin include antimicrobial lipids, acids, hydrolytic enzymes, antimicrobial peptides and macrophages. The cells of the epidermis enable induction of the adaptive immune response (Antigen presenting l Langerhans cells, cytokine and growth factor producing keratinocytes; mast-cells containing cytokines and enzymes and lymphocytes producing cytokines and antibodies). (Proksch *et al.* 2008)

2.5.3 Cutaneous wound healing

Wound healing is a well-orchestrated interplay of cells and mediators aiming at rapid restoration of the tissue structure. In brief, wounding induces the formation of a blood clot that fills the tissue defect and initiates the signalling events that lead to the recruitment of inflammatory cells. During inflammation the wound is cleaned from foreign material. The regeneration of tissue starts to take place, as platelets and inflammatory cells produce a plenary of cytokines and growth factors. Fibroblasts produce collagen rich provisional matrix that together with regenerating capillaries make up the granulation tissue. Simultaneously, the epidermal cells proliferate and migrate through the provisional matrix in order to create a new epidermal sheet and basement membrane – a process called re-epithelisation. Remodelation occurs when proteolytic and apoptotic mechanisms remove the excess matrix along with synthesis. As a result, the tissue structure and function of the skin is restored to the best of its ability and the rest is replaced with collagenous scar ensuring tissue integrity. New interactions that overlap the traditionally considered distinct phases of hemostasis, inflammation, proliferation and maturation have been discovered, which increases our understanding of the complexity of this process essential to life. (Broughton *et al.* 2006) The following chapters outline the processes of extracellular matrix remodellation, angiogenesis and re-epithelisation in skin wound.

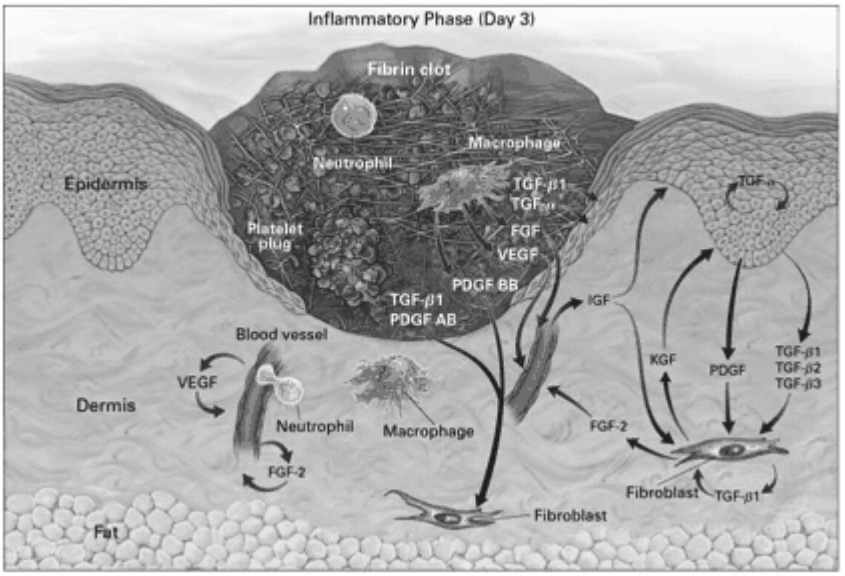

Fig. 14. A cutaneous wound three days after injury. Growth factors thought to be necessary for cell movement into the wound are shown. TGF-ß1, TGF-ß2, and TGF-ß3 denote transforming growth factor ß1, ß2, and ß3, respectively; FGF fibroblast growth factor; VEGF vascular endothelial growth factor; PDGF, PDGF AB, and PDGF BB platelet-derived growth factor, platelet-derived growth factor AB, and platelet-derived growth factor BB, respectively; IGF insulin-like growth factor; and KGF keratinocyte growth factor. (Reprinted by permission from Singer & Clark 1999. Copyright © [1999] Massachusetts Medical Society. All rights reserved.).

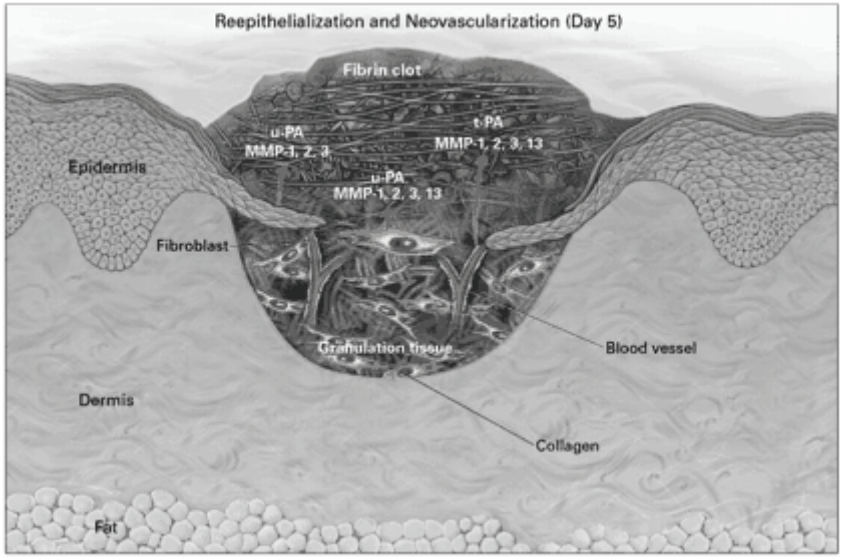

Fig. 15. A cutaneous wound five days after injury. Blood vessels are seen sprouting into the fibrin clot as epidermal cells resurface the wound. Proteinases thought to be necessary for cell movement are shown. The abbreviation u-PA denotes urokinase-type plasminogen activator; MMP-1, 2, 3, and 13 matrix metalloproteinases 1, 2, 3, and 13 (collagenase 1, gelatinase A, stromelysin 1, and collagenase 3, respectively); and t-PA tissue plasminogen activator. (Reprinted by permission from Singer & Clark 1999. (Copyright © [1999] Massachusetts Medical Society. All rights reserved.))

Extracellular matrix remodellation and angiogenesis

Timely organization of extracellular matrix deposition and degradation characterises the building of new stromal tissue and is a well-orchestrated interplay between fibroblasts, macrophages and endothelial cells. The role of the different cells have been described by Singer*"The macrophages provide a continuing source of growth factors necessary to stimulate fibroplasia and angiogenesis; the fibroblasts produce the new extracellular matrix necessary to support cell ingrowth and blood vessels carry oxygen and nutritiens necessary to sustain cell metabolism"*(Singer & Clark 1999).

The fibroblasts move to the wound by lamellopodial crawling, directed by contact guidance of matrix proteins and chemotactic stimuli. The factors chemotactic to fibroblasts include FGF from *endothelial cells*, TGF-β and PDGF from the

platelets and keratinocytes and TGF-β from the *macrophages*. The proliferation of fibroblasts is in turn induced by FGF, EGF, PDGF, TGF- α, TGF-β and TNF-α. The fibroblasts enhance the signals by auto- and paracrine stimulation with more PDGF and TGF-β. As a result, fibroblasts accumulate in wound tissue (Broughton *et al.* 2006).

Once in wound, fibroblasts start to express matrix proteins on the initial matrix composed of the fibrin and fibronectin of the clot. First a loose matrix rich in fibronectin and proteoglycans is created. Collagen III production predominates early, and as collagen I accumulates, the wound braking strength increases (Witte & Barbul 1997).

Simultaneously, the degradation of provisional matrix takes place. Fibroblasts produce proteases like plasminogen activators and matrix metalloproteinases in order to cut their way into the provisional matrix.

The mechanical forces and contact to matrix proteins through integrin adhesions modulate the fibroblast movement and protein expression profile. In three dimensional cell cultures, the lack of mechanical load results in phenotype characterized by expression of inflammatory mediators and proteases and low proliferation. Then again, mechanical loading creates a proliferating, matrix producing fibroblast that is characterised by α-smooth muscle actin positive stress fibres (Eckes *et al.* 2006). In vivo the interactions are much more complex, with other cells and mediators modulating the response. However the mechanical milieu can play a more significant role than previously acknowledged. Early in wound healing, the matrix is compliant allowing migration of fibroblasts and inflammatory cells. However, as collagen accumulates, stiffness and cell to matrix adhesions increase and tensile forces are created within fibroblasts, which in turn cause a shift in phenotype to fibrogenic and contractile myofibroblast. (Broughton *et al.* 2006) TGF-β is the main cytokine augmenting the fibrogenetic response in fibroblasts and is produced by macrophages and platelets. TGF-β increases fibroblast differentiation to myofibroblast phenotype. As ECM becomes organised and collagen I accumulates, the tensile forces within ECM diminish. The presence of collagen I diminishes α-smooth muscle actin in fibroblasts. As a result of extracellular matrix and fibroblast relaxation, apoptosis of excess fibroblasts seem to occur —an event that TGF-β1 stimulation is unable to overcome. (Chipev & Simon 2002). Accordinly collagen synthesis diminishes. Within the forthcoming months the initial collagen is replaced by thicker fibres that are organised along the stress lines (in contrast to deposition parallel to the skin in granulation tissue) (Witte & Barbul 1997).

Re-epithelisation

Re-epithelisation begins within the first hours from injury in order to re-establish the epidermal protective barrier. The keratinocytes on wound edges and in skin appendages undergo alterations that enable migration to the wound tissue: Hemidesmosomal junctions connecting keratinocytes to basement membrane are disassembled and intercellular connections are reorganised by up-regulating and changing the integrin profile (Cavani *et al.* 1993, Jacinto *et al.* 2001). Integrins are transmembrane cell surface receptors that connect the cytoskeletal structures to matrix proteins and thereby enable the movement of keratinocytes. Integrins are heterodimers of various α and β chains and each combination has unique binding specifity and signalling properties (Santoro & Gaudino 2005).

In addition to alterations wounding causes to cell-cell and cell-matrix contacts, the signals promoting migration include the growth factors from activated platelets and macrophages (TGF-α, EGF) as well as fibroblasts (Keratinocyte growth factors and IL-6). The migrating keratinocytes cut their way between the clot and regenerate granulation tissue by producing plasminogen activator and matrix metalloproteinases. To compensate the cell loss keratinocytes behind the migrating cells begin proliferating. After a monolayer of cells is formed, a new basement membrane is deposited from the wound margins to the center. (Santoro & Gaudino 2005)

2.5.4 Cutaneous wound healing in sepsis

Wound healing seems to be defective in sepsis. The wound breaking strength and collagen content have been shown to be decreased in experimental animals with sepsis (De Haan BB 1974; Greenhalgh DG 1987, Stamm J 2000). In septic mice, decreased wound collagen content and delayed wound re-epitheliasation were observed (Rico *et al.* 2002). In septic rat model, the breaking strength of incisional wounds was shown to be decreased, and administration of TNF antagonist significantly improved incisional wound strength in that study (Cooney R 1997; Maish GO 3rd 1998). However, the results must be interpreted with caution as human wound healing, especially in clinical sepsis, presumably differs from that of experimental animal studies.

Human studies on collagen synthesis in critical illness are few. By using a subcutaneously implanted tube in the intact upper arm, Clark and colleagues have shown that the levels of hydroxyproline, a collagen-specific aminoacid, are

decreased in septic major trauma (Clark *et al.* 2000). In addition, reduced skin collagen synthesis in intact skin remote to operative wound following intra-abdominal surgery has been reported (Ihlberg *et al.* 1993).

2.5.5 Skin microcirculation in sepsis

Skin microcirculation supplies oxygen and nutritients to the skin and removes waste products. The composition of plasma, endothelial barrier and interstitium with lymphatic drainage regulate together the forces that enable this normally well balanced exchange.

Septic microcirculation is characterised by increased permeability and severe alterations in the composition of blood. Presumably, the composition of the surrounding interstitium also impacts the functionality of the microcirculation, but studies concentrating on this issue in septic microcirculation are lacking. However, interesting insights can be obtained from studies on burn trauma induced systemic inflammation. Postburn edema in non-burned soft tissues is suggested to result from generalized release of inflammatory mediators and oxygen radicals that would lead to increased vascular permeability. Another important phenomenon seems to be the decrease and fragmentation of interstitial protein and resulting increase in the ease of fluid accumulation in the interstitium (Demling 2005).

Factors affecting the state of microcirculation are suggested to alter in time and between organs, which makes the microcirculatory disturbance in sepsis a very complex entity. There are also different methods for studying microcirculation in clinical setting. The elegant methods of orthogonal polarization spectral (OPS) imaging and side stream dark field (SDF) imaging reach only to a depth of 1mm and are most convenient in studying thin mucosae.

Skin in sepsis has not been studied by these relatively novel methods. Sublingual imaging of microcirculation in sepsis has provided important insights into the septic microcirculation. Even when the macrocirculatory parameters are corrected, persistent sublingual microcirculatory alterations in septic shock are associated with poor prognosis (Sakr *et al.* 2004). Recently it has also been shown that improvement in sublingual microcirculatory parameters during the first hours after intensive care unit admission was associated with the improvement of organ function measured with SOFA in the first 24 hours (Trzeciak *et al.* 2008).

Previously the skin microcirculation in sepsis has been studied with laser – doppler flowmeter, which detects circulating red blood cells to the depth of 1–1.5mm. Unlike OPS it cannot distinguish the calibre or direction of the vessel, but

summarises the whole circulation at a certain depth. In skin the laser Doppler method reaches only the arterioles, capillaries and postcapillary venules of the upper dermal vascular plexus and thus is quite an adequate method in studying the skin microcirculation (Choi & Bennett 2003). Previously it has been noted in small studies with sepsis patients that cutaneous microcirculation in forearm is increased. (Sair *et al.* 2001, Young & Cameron 1995)

2.5.6 The suction blister method in studying wound healing

The suction blister method was developed by Kiistala (Kiistala 1968). A prolonged suction separates the epidermis from the dermis underneath the basal cell layer just above the layer of lamina densa of the basement membrane. The blister becomes filled with fluid closely resembling interstitial fluid (Vermeer *et al.* 1979). Previously the method has been used for studying wound healing in settings focusing on the basic biology of wound healing, healing of burn injuries and effects of jaundice and diabetes on epidermal wound healing (Koivukangas *et al.* 1999, Koivukangas *et al.* 2005, Leivo *et al.* 2000, Svedman *et al.* 1991). The suction blister method enables the examination of wound healing non-invasively in vivo. There are methods for studying re-epithelisation, blood flow and standardised biochemical analyses of proteins in suction blister fluid.

Re-epithelisation can be studied by measuring water evaporation repeatedly from the suction blister area. After the blister roof is removed the rate of water evaporation is 15–20 fold compared to skin with intact epidermal barrier. As keratinocytes migrate in order to regenerate the epidermal barrier, the water evaporation decreases (Leivo *et al.* 2000). TEWL is a measure of the inside-out barrier function of the skin, e.g. a measure of fluid loss rather than a measure for outside-in trafficking like bacterial invasion.

Inflammation causes increase in wound blood flow as vasodilative agents are released, among others nitric oxide. Thus the level of blood flow is an indirect measure of inflammation. Red blood cell velocities and density in the outer plexus of dermis can be measured by using laser Doppler flowmeter. The laser beam penetrates to a depth of approximately 1mm and thus reaches the outer dermal vascular plexus. The laser light is shifted in frequency when it is scattered by moving red blood cells according to Dopplerprinciple. The backscattering light thus contains the information of red blood cell velocity and density and is

detected photoelectrically. The results are expressed as perfusion units which are arbitrary (Choi & Bennett 2003).

3 Aims of the study

The aim of this study was to reveal new insights into the pathology of tissue healing and in particular connective tissue metabolism in severe sepsis using skin as an example of one affected organ.

In detail the aim was to test the following hypotheses:

1. The levels of systemic collagen synthesis and degradation are altered in severe sepsis and is associated with mortality and severity of septic organ failures. (I)
2. Epithelisation is delayed in a skin wound in severe sepsis and the inflammatory phase of wound healing is affected by systemic inflammation. (II)
3. Intact skin collagen synthesis is altered in severe sepsis. (III)
4. Matrix metalloproteinases play a role in sepsis patophysiology and are associated with mortality and severity of organ failures (IV)

4 Patients and methods

4.1 Design and setting

The study was a prospective observational case control study describing alterations in markers of connective tissue metabolism in severe sepsis.
It was divided into four substudies hereafter referred to as studies I-IV corresponding to the list of original publications. All the studies were approved by The Ethics Committee of Oulu University Hospital. The studies were conducted in the mixed adult tertiary level intensive care unit, Department of Anaesthesiology, Oulu University Hospital in collaboration with the departments of Dermatology, Surgery, Clinical Chemistry and Dentistry of Oulu University Hospital. The laboratory work was done in the research laboratory of Clinical Chemistry in studies II and III and in the research laboratory of the Department of Diagnostics and Oral Medicine in study IV.

4.2 Patients

During a 1.5 year period, from 10th May 2005 to 15th December 2006, 1361 patients admitted to the Intensive care unit were screened for eligibility for the study. Of these patients 238 fulfilled the inclusion criteria, which was the diagnosis of severe sepsis according to ACCP/SCCM criteria (Bone *et al.* 1992). The exclusion criteria included other reasons for changed collagen synthesis or degradation and were the following: age under 18 years or over 80 years, bleeding disorder not related to sepsis, surgery not related to sepsis, malignancy, chronic liver disease, chronic kidney disease, immunosupression and cortisone treatment for other reasons than septic shock. Also, long distance/inability to travel to control visits and early death/transport were reasons that led to drop out. Altogether 172 patients had to be excluded. If a time window of 48 hours from the diagnosis of severe sepsis was closed before sampling could be initiated, a patient was no longer considered to be eligible for the study. Informed consent from the patient or next of kin and timely sampling could be obtained from 44 patients. Due to technical problems physiological measurements from suction blisters could be obtained only from 35 patients. Thus the patients in studies I, III and IV consisted of 44 and in study II of 35 adult patients with severe sepsis. Additionally, 15 healthy adults were used as control subjects in all substudies.

4.3 Clinical data

In all substudies the following data were prospectively collected from the hospital records and the intensive care unit's data management system (Centricity Critical Care Clinisoft, GE Healthcare, Helsinki, Finland): age, sex, body mass index, type of intensive care unit admission (surgical or medical), reason for intensive care unit (ICU) admission, infection focus and pathogen, presence of chronic underlying diseases, Acute Physiology and Chronic Health Evaluation (APACHE) II score (Knaus *et al.* 1981, Knaus *et al.* 1985) on admission and evolution of daily organ dysfunctions assessed by daily Sequential Organ Failure Assessment (SOFA) (Vincent *et al.* 1996, Vincent *et al.* 1998), length of ICU and hospital stay, hospital and 30-day mortality and information of medications and fluid therapy given. In addition lactate levels from laboratory parameters were collected.

The median age of the 44 study patients was 63 years (25^{th}-75^{th} percentile 56–71) and the median age of the controls was 60 years (25^{th} to 75^{th} percentile 56–68). The median APACHE II score of the patients on admission was 26 (22–30), and mortality over 30 days was 25%. (I, III, IV) The median age of the 35 patients included in substudy II was 63 years (25^{th}-75^{th} percentile 57–69) and 30 day mortality was 29% (II). (Table 9) Detailed patient characteristics are published in the substudies (I-IV).

Table 9. Basic charachteristics of the study patients.

Characteristics	Study II	Studies I, III, IV
Patient N	35	44
Male N	22 (63%)	29 (66%)
Age, median	63 (57 to 69)	63 (56 to 71)
APACHE II on admission	24 (22 to 29)	26 (22 to 30)
Multiple organ failure	23 (66%)	30 (68%)
Maximum SOFA score	10 (8 to 14)	10 (7 to16)
30-day mortality	10 (29%)	11 (25%)

4.4 The time course of the collection of the data

Table 10. The time course of the collection of study data.

Sampling and bed-side measurements										
Study days	1	2	3	4	5	6	7	8	9	10
Induction of suction blisters (suction blister fluid obtained)	x				x					
Measurements of TEWL[1] and BF[2] from the 1st suction blister	x				x					
Measurements of TEWL[1] and BF[2] from the 2nd suction blister					x			x		
Venous blood samples (number of samples obtained/day)	4	4	1	1	1	1	1	1	1	1

[1] TEWL = Transepidermal water loss, [2] BF = Blood flow

4.5 The suction blister method

The suction blister method was used in substudy II for the assessment of wound healing response in sepsis, in particular re-epithelisation and inflammation, and in substudies III and IV for collection of the suction blister fluid for procollagen propeptide, blister fluid protein and matrix metalloproteinase analysis. Suction blisters were induced within 48 hours of the diagnosis of severe sepsis (early blister) and four days later (late blister). Additively, blisters were induced on survivors after three and six months. Only one set of blisters were induced on the controls. The suction blister device (Dermovac blistering device, Mucel Ky, Nummela, Finland) (Figure 17) was applied on intact abdominal skin and a negative pressure of 250mmHg was conducted to the blister chambers. After 30 min the pressure was increased to −150mmHg and the blisters were allowed to develop for another 15–30 minutes. The skin area was slightly warmed with incandescent lamp. Instantly after the blisters were fully developed, the blister fluid was collected. The blister fluid was collected with an 18G needle and syringe and was frozen to −70 °C until analysis.

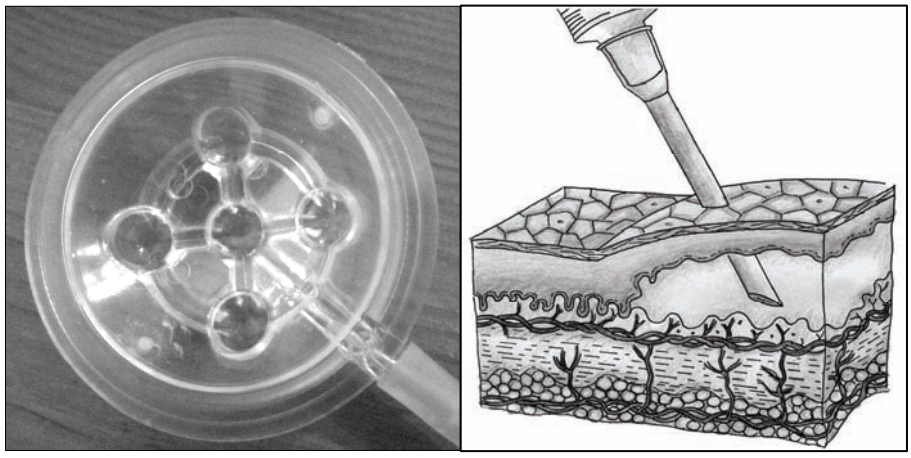

Fig. 16. [left] Photograph of the suction blister device. The blistering device used in this study was 50mm in diameter and contains five blister chambers 6 mm in diameter. [right] Schematic drawing describing the suction blister method: the blister is formed underneath the basal cell layer just above the layer of lamina densa of the basement membrane. After blister formation the fluid was collected and blister roofs removed.

4.5.1 Measurements of water evaporation and blood flow (II)

The suction blister method enables the investigation of wound healing in vivo as an experimental wound of standard size and depth is created. There are applications for studying two main phenomena of wound healing, namely re-epithelisation and inflammatory response.

Since the epidermis regulates the water evaporation from the skin, transepidermal water loss (TEWL) increases when the epidermis is removed. This was measured in our study after the suction blister fluid was collected and the blister roofs removed. The device used was VapoMeter (Delfin Technologies Ltd, Kuopio, Finland. http://www.delfintech.com), which measures the amount of water loss in grams per square metre. There is a cylindrical chamber in the head of the VapoMeter where the sensors for humidity and temperature are located. VapoMeter on the skin forms a closed chamber in which the system automatically calculates the evaporation rate from the increase in relative humidity. TEWL was measured instantly after induction of the blisters on day one and five, and a new measurement was done after five days of healing, that is on days five and eight. During healing the epidermal cells migrate into the wound area and as a result

water evaporation decreases. All five blisters were measured and the mean value was calculated and reported. In addition, we measured, simultaneously with blister measurements, the transepidermal water loss from intact abdominal skin.

The blood flow in the wounded area can be measured using a Laser-Doppler flow meter (Periflux Pf1,Perimed KB, Stockholm, Sweden), and can used as a measure of inflammation. The laser beam penetrates into the skin a to depth of about 1mm. The laser Doppler reaches only the superficial vascular plexus of the the skin, which lies beneath the dermo-epidermal junction (Choi & Bennett 2003). All the five blister wounds were measured and the mean was calculated and reported. Measurements are expressed as perfusion units, which is arbitrary. Blood flow was measured simultaneously with TEWL measurements.

Between the measurement points the wounds were covered with an air and water vapour permeable, self-adhesive dressing between the study days (Mepore, Mölnlycke Health Care AB, Göteborg, Sweden). All blister inductions and measurements were made in the same circumstances, i.e. air temperature and all blister wounds were induced and measurements were performed by MK and FG.

4.5.2 Procollagen propeptide measurements (I,III)

The blister fluids were stored at −70 °C until analysis. The PINP and PIIINP concentrations were measured using radioimmunological assays (Orion Diagnostica, Espoo, Finland) (Oikarinen *et al.* 1992a, Melkko *et al.* 1996).

The first blood samples for procollagen types I and III aminoterminal propeptides (PINP, PIIINP) and the cross-linked telopeptide of type I procollagen (ICTP) were obtained instantly after study admission. The blood samples were collected at 6-hr intervals up to 48 hours and thereafter once a day for 10 days. If a patient died or was discharged from the hospital, the follow-up was discontinued earlier. Blood samples were collected once in the control group. After centrifugation, the serum was stored at −70 °C. PINP, PIIINP and ICTP were analyzed using radioimmunological assays (Orion diagnostica, Espoo, Finland). Reference values are published elsewhere (Risteli & Risteli 2002). The coefficients of variation (CV) of the ICTP method were between 3% and 8% for a wide range of concentrations. For serum intact PINP assay, the inter- and intra-assay of CVs were 3.1–9.3% for values within the reference intervals. For serum PIIINP assay the inter- and intra-assay of CVs were 3.0–7.2% for values ranging from 2.7–12.2 µg/L.

4.5.3 Measurement of the overall protein concentration in suction blister fluid (II)

Protein concentration of the blister fluid was measured using colorimetric DC Protein Assay by Bio-Rad (Bio-Rad Laboratories Inc., California, U.S.A). The absorbance was measured using a wavelength of 650nm.

4.5.4 Measurement of the lactate concentration (III)

The lactate concentrations were obtained by using amperometric lactate biosensor (Rapidlab 865, Siemens Healthcare Diagnostics). Arterial blood was analyzed instantly after sampling, or cooled down to 5 °C until analysis within 30min from sampling. The analyses were done at 37 °C.

4.5.5 Measurements of MMP-2 and MMP-9 by gelatin zymography (IV)

To measure gelatinase activity of matrix metalloproteinases 2, 8 and 9, 1µl of serum or 2µl of suction blister fluid was added to 10% SDS-PAGE containing 1% of fluorescently labelled gelatin. Some suction blister fluid samples were preconditioned by incubating them at 37 °C for 1 h with 2 mM APMA (4-aminophenylmercuric acetate, which is known to activate MMPs, Sigma). The APMA treatment was stopped by adding the electrophoresis sample buffer. After electrophoresis, the gels were washed with 2.5% TritonX-100 buffer to remove SDS and renature the MMPs in the gels. Then the gels were incubated in a developing buffer overnight to induce gelatin lysis by renatured MMPs. The gelatinolytic activity was observed under long-wave UV-light and then the gels were stained with 0.5% Coomassie Brilliant Blue R-250. The intensities of the bands were quantified using optical densitometry and Quantity one software (Bio Rad Model GS-700 Imaging Densitometer, Bio-Rad, Richmond, CA, USA). The intensity is expressed as densitometric units (dU).

4.5.6 Immunofluorometric assay of MMP-8 (IV)

The MMP-8 concentrations were determined by a time-resolved immunofluorometric assay (IFMA). The monoclonal MMP-8 specific antibodies 8708 and 8706 (Medix Biochemica, Kauniainen, Finland) were used as a catching antibody and a tracer antibody, respectively. The tracer antibody was labeled

using europium-chelate. The assay buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM $CaCl_2$, 50 µM $ZnCl_2$, 0.5% BSA, 0.05% sodium azide and 20 mg/l diethylenetriaminepentaacetic acid (DTPA). Samples were diluted in assay buffer and incubated for one hour, followed by incubation for one hour with tracer antibody. Enhancement solution was added and after 5 min fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The specificity of the monoclonal antibodies against MMP-8 corresponded to that of polyclonal MMP-8.

4.6 Statistical methods

The software used for statistical analysis was SPSS (version 15.0 (I-III) and 16.0 (IV), SPSS Inc., Chigaco, IL, USA).

The summary statistics are presented in absolute numbers with percentages, means with standard deviation or medians with 25^{th} to 75^{th} percentiles.

Statistical significance was defined as P-value under 0.05. Two tailed P-values are reported in all substudies.

Chi-Square test and Fisher's exact test were used to analyze categorical variables in all substudies

Mann-Whitney U-test and Student's T-test were used to compare continuously distributed data in two independent groups.

Receiver Operating Characteristics (ROC) curve was used to analyse post-test probability of organ failures after PINP and PIIINP measurements in substudy I.

The linear mixed model was used for repeated measurements analyses when comparing MODS and MOF patients in substudy IV.

Spearmans rank order was used to test the relations between variables in all substudies.

The Wilcoxon signed rank test was used to analyze the differences between the different times of measurements in substudy I.

5 Results

5.1 Restoration of epidermal barrier and microcirculatory response in experimental blister wounds (II)

Restoration of epidermal barrier measured as decrease in transepidermal water loss (TEWL) was slower in patients with severe sepsis (ΔTEWL= $TEWL_{after\ induction} - TEWL_{fourth\ day}$). The mean decrease was 56 g/m^2 in the septic group and 124 g/m^2 in the control group. The same trend was seen in the late wound, for which the decrease was 77 g/m^2 (SD 63) in the septic group and 124 g/m^2 in the control group (P = 0.091) (II).

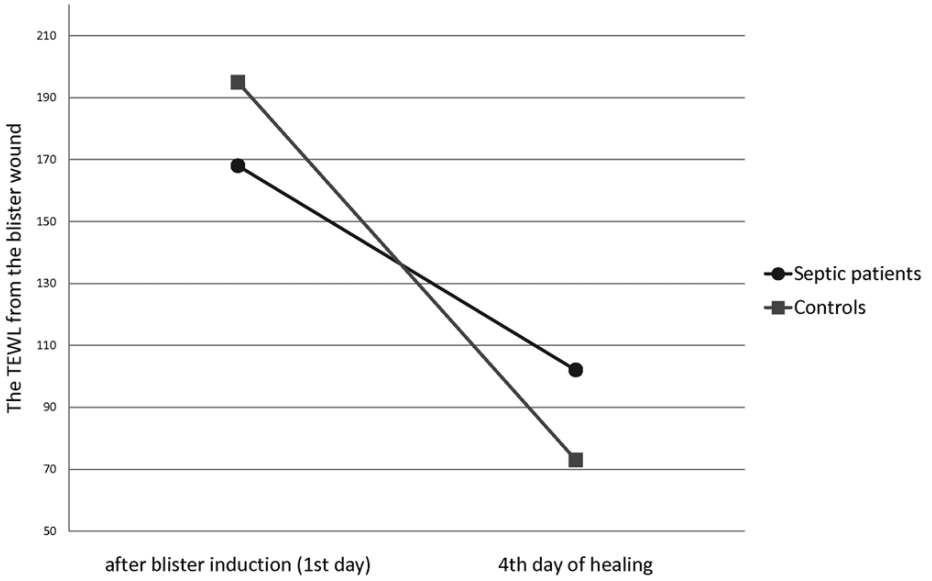

Fig. 17. The decrease of transepidermal water loss (g/m^2 per hour) from the first to the fifth day of the early wound. The decrease was lower in the septic group compared with the controls (P = 0.004) (II, Figure 1).

Blood flow measured with laser-doppler flowmeter in patients with sepsis was not different from the controls. However, on the fifth day the septic patients had higher values both in the early blister, which was induced four days earlier, and in the freshly induced late blister. Also, in the second measurements of late blister after four days of healing, the blood flow was higher than in the controls. (Table 11)

Table 11. Blood flow measured from the blister wound.

Group	Day 1 early wound		Day 5 early wound		Day 1 late wound		Day 5 late wound	
	BF	SD	BF	SD	BF	SD	BF	SD
Sepsis	76	49	110	67	101	50	110	58
Control	51	11	47	24	51	11	47	24
	P = 0.273		P = 0.001		P = 0.001		P = 0.005	

Values other than *P* values are expressed in perfusion units. BF, blood flow; SD, standard deviation.

On the first day there were no differences in mean blood flow as measured from the intact abdominal skin in the septic group (15 units, SD 12) and in the control group (14 units, SD 9). However, on the fifth day the mean blood flow measured from intact skin was higher in the septic group (24 units, SD18) compared with the controls (6 units, $P < 0.000$) (II, Figure 4).

5.2 Collagen synthesis in severe sepsis at the whole-body level and in skin (I, III)

To evaluate the systemic and skin collagen synthesis in severe sepsis, repeated measurements of serum and suction blister fluid collagen I and III propeptides were made.

In serum the median PIIINP concentration was higher in sepsis patients than in the controls already on the first day (8.8 μg/L v.s. 3.0 μg/L, $P < 0.001$). The difference was statistically significant in all ten days studied and even at 3 and 6 moths (P-values for comparisons up to ten days < 0.001 each and at 3 and 6 months < 0.01). Even the median of minimum PIIINP values of the patients was lower than the median PIIINP value of the controls (7.2 μg/L vs. 3.0 μg/L, $P < 0.001$). The subgroups of non-surviving patients and patients with multiple organ failure had higher levels than surviving patients and patients with less severe organ failures, respectively (I, figures 1 and 4). Surviving patients had elevated PIIINP values in the light of the laboratory reference values even at three and six months after severe sepsis (I).

On the other hand, the median of PINP concentration of the patients did not differ from controls on either of the days studied (I). Even though the PINP values of the patients with severe sepsis remained within the laboratory reference values, the patients with MOF had higher values than the patients with MODS (79.8

vs.40.4, $P = 0.007$). Maximum PINP values in surgical and non-surviving patients did not differ from the values of medical and surviving patients, respectively (I).

An interesting finding was that the PIIINP/PINP ratio was close to thirty percent during the ten study days apart from the value of the controls (6.4%) (Figure 19), but had returned to normal at 3 and 6 months.

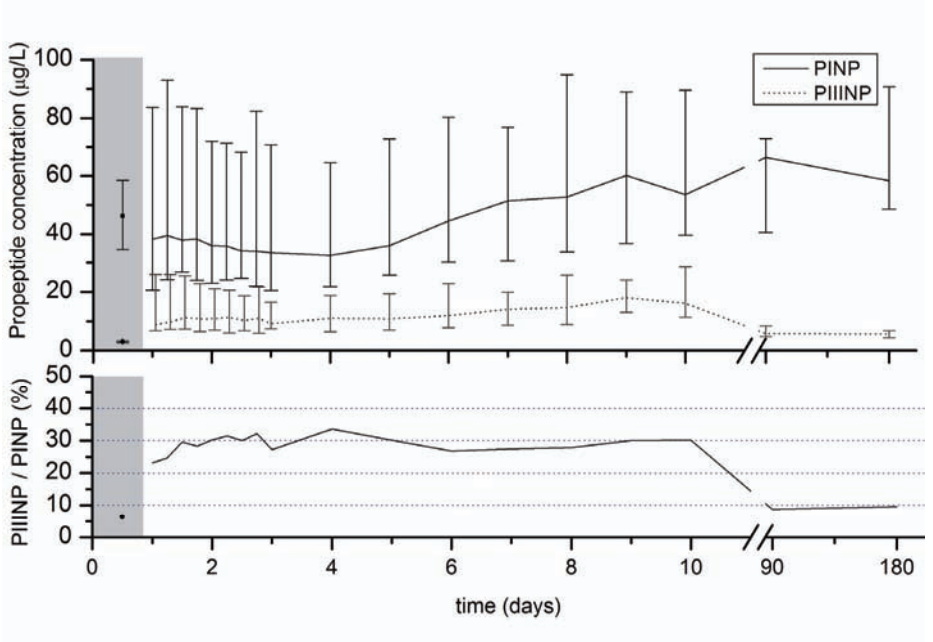

Fig. 18. Procollagen III and I concentrations in the serum of patients with severe sepsis. Control values are drawn on light grey background (PINP above PIIINP). The vertical lines represent the 25th to 75th percentile.

The markers of collagen synthesis correlated positively with 30-day mortality and maximum SOFA scores (Table 12).

Table 12. Correlations of serum PINP and PIIINP with 30-day mortality and maximum of total SOFA scores.

	30-day mortality	Maximum total daily SOFA score
PIIINP day 1	0.62***	0.62***
PIIINP maximal levels	0.47***	0.57***
PINP day 1	0.25	0.59***
PINP maximal levels	0.37**	0.59***

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

In suction blister fluid, on the other hand, the PIIINP and PINP levels during sepsis were found to be lower than in the controls. Also, the overall protein concentration was found to be lower (III, figure 1) and thus when calculating the results towards protein instead of volume, the differences were attenuated (Table 13).

Table 13. PINP and PIIINP in suction blister fluid in severe sepsis.

Procollagen Propeptides	µg/L			µg/g protein		
	Patients	Controls	P-value	Patients	Controls	P-value
PIIINP early blister,	40.8	69.6	0.028	3.4	3.8	0.721
median [25th-75th percentile]	[22.2–77.1]	[47.2–104.7]		[2.1–8.7]	[2.4–5.5]	
PINP early blister,	69.9	243.2	< 0.001	6.0	13.5	0.07
median [25th-75th percentile]	[32.4–112.7]	[182.3–342.9]		[3.2–12.3]	[9.0–18.5]	
PIIINP late blister,	38.8	69.6	< 0.001	3.5	3.8	0.953
median [25th-75th percentile]	[19.9–68.5]	[47.2–104.7]		[2.1–6.4]	[2.4–5.5]	
PINP late blister,	90.0	243.2	< 0.001	8.9	13.5	0.05
median [25th-75th percentile]	[35.1–138.8]	[182.3–342.9]		[2.9–15.3]	[9.0–18.5]	

µg/l = micrograms procollagen propeptide in a liter od suction blister fluid,

µg/g =Micrograms procollagen propeptide in a gram of suction blister fluid protein

The PIIINP/PINP ratio in the suction blister fluid of the controls was 0.29 [0.25–0.32] which was approximately twice as high in patients in the early blister (0.59 [0.41–0.71]) and in the late blister (0.46 [0.34–0.71])

5.3 Markers of collagen breakdown (I, IV)

ICTP levels were higher in the septic patients compared with the controls (19.4 µg/L [25th to 75th percentile 12.0 to 29.8] vs. 4.1 µg/L [3.4 to 5.0], $P < 0.001$) on the first day. The maximum and minimum values over the 10-day period were clearly higher in comparison with the control value (31.3 µg/L [18.3 to 49.0], $P < 0.001$ and 16.0 µg/L [10.5 to 26.5], $P < 0.001$, respectively). Non-surviving patients and patients with MOF had higher values than surviving patients and patients with MODs respectively, but surgery and cortisone supplementation therapy did not seem to have an impact on the results (II). Maximum and day one ICTP levels correlated positively with maximum total SOFA-scores (II).

MMP-8, which is known to cleave collagen I, seems to increase along with the increasing ICTP-levels (Figure 20). However, no statistically significant

correlations were found between ICTP and MMP-8 values of the patients. At three and six months the levels of the surviving patients were near to normal.

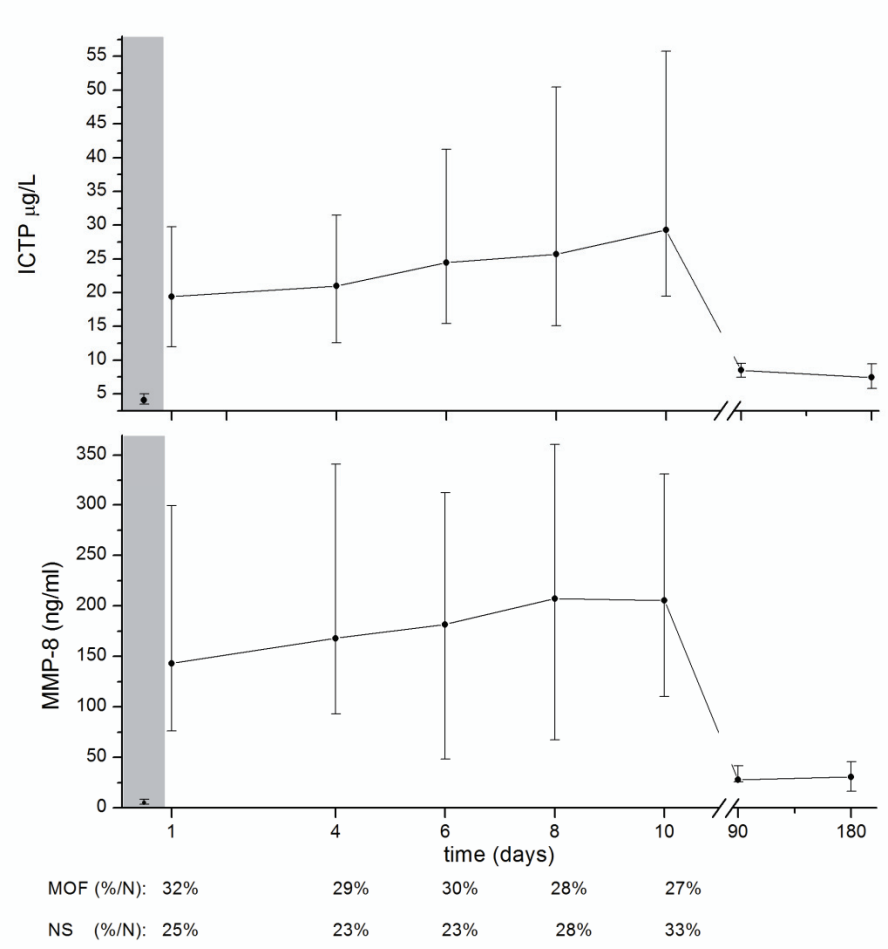

Fig. 19. The development of ICTP and MMP-8 levels in patients in severe sepsis. The control values are presented on the left on light grey background. The proportion of MOF and non-surviving (NS) patients are added below (A similarly increasing trend in ICTP and MMP-8 values also in subgroups of MODS, MOF, surviving and non-surviving patients).

5.4 Balance of collagen I synthesis and breakdown (I)

PINP/ICTP ratio describes the balance of collagen I production and MMP-mediated collagen I degradation. In the serum of the controls the median of the ratios was 10.3 [25th to 75th percentile 8.6–13.7]. In comparison with this value, the values of the patients with severe sepsis were much lower during the ten study days ($P < 0.001$, for each comparison) and at three months ($P = 0.001$). At six months the ratio seemed to be closer to that of the controls ($P = 0.134$). (Figure 21). Comparing the different patient groups, no differences in PINP/ICTP ratio was found (MODS-MOF, surviving-non-surviving, hydrocortisone-no hydrocortisone, surgical-medical)

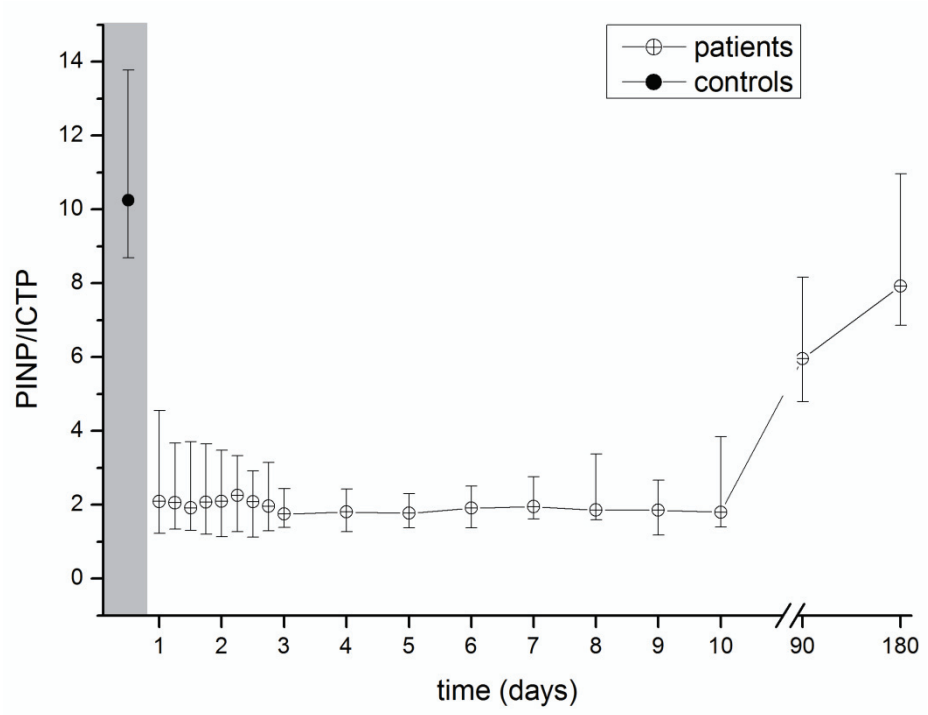

Fig. 20. PINP/ICTP ratio of patients and controls. The symbols describe the median values and the vertical lines 25th to 75th percentiles. The values of the controls are presented on light grey background.

5.5 Matrix metalloproteinases -2, -8 and -9 in serum and skin blister fluid in severe sepsis (IV)

In serum MMP-8 and MMP-2 levels were higher in sepsis than in healthy controls, whereas MMP-9 levels tended to be lower (Figure 22). Both pro and active forms of MMP-2 and MMP-9 were measured. The form of MMP-2 spliced to active (62kDa) could not be detected in the serum samples in patients and controls and the 82kDa MMP-9 form was detected in few samples (IV). Serum levels of the MMPs studied did not differ in surviving and non-surviving patients, whereas in comparing MODS and MOF, it was found that MMP-8 increased more in time in MOF (IV, figure 4)

In blister fluid the levels of MMP-2 and MMP-8 were elevated, whereas the MMP-9 was low in both early and late blisters (Figure 22). The form spliced to active conformation, the 62 kDa MMP-2, was found in all patients with severe sepsis on the first day (153.1 dU [53.2–373.9]) and on the fifth day (127.4 dU [47.4–318.2]), but not in the controls (IV, figure 2). The 92 kDa proMMP-9 was lower on both the first and fifth day in patients with severe sepsis in comparison with the controls (Figure 1.). The 82 kDa MMP-9, the form spliced to active conformation, was found in blister fluid samples of five patients out of 44 on the first day and of five patients out of 38 patients on the fifth day, but not in the control samples.

Comparing non-survivors to survivors, it was observed that the nonsurvivors had higher levels of proMMP-2 on both days. Also, the form spliced to active conformation was higher on the fifth day in non-survivors. There were no differences in MMP-8 levels of suction blister fluid in these subgroups, whereas proMMP-9 was higher in non-survivors on day one (Table 14).

Table 14. The levels of matrix metalloproteinases -2, -8 and -9 in skin blister fluid in severe sepsis.

	Non-survivors	Survivors	p-value
Day 1. (early blister)			
MMP-8 (ng/ml)	28.8 [8.2–84.7]	12.8 [5.2–52.8]	0.47
MMP-2 72*kDa* (du)	1132.2 [922.1–1405.1]	701.9 [604.7–941.1]	0.001*
MMP-2 62*kDa* (du)	98.3 [39.3–237.1]	351.7 [189.1–598.6]	0.13
MMP-9 *92kDa* (du)	365.43 [221.1–478.3]	102.8 [60.8–273.7]	0.005*
Day 5. (late blister)			
MMP-8 (ng/ml)	13.5 [6.6–47.1]	20.7 [4.5–67.4]	0.84
MMP-2 72*kDa* (du)	1153.9 [801.9–1349.4]	735.9 [627.4–888.6]	0.01*
MMP-2 62*kDa* (du)	425.8 [201.6–474.9]	84.9 [44.9–243.4]	0.02*
MMP-9 92*kDa* (du)	151.60 [37.5–231.5]	127.9 [47.8–283.4]	0.84

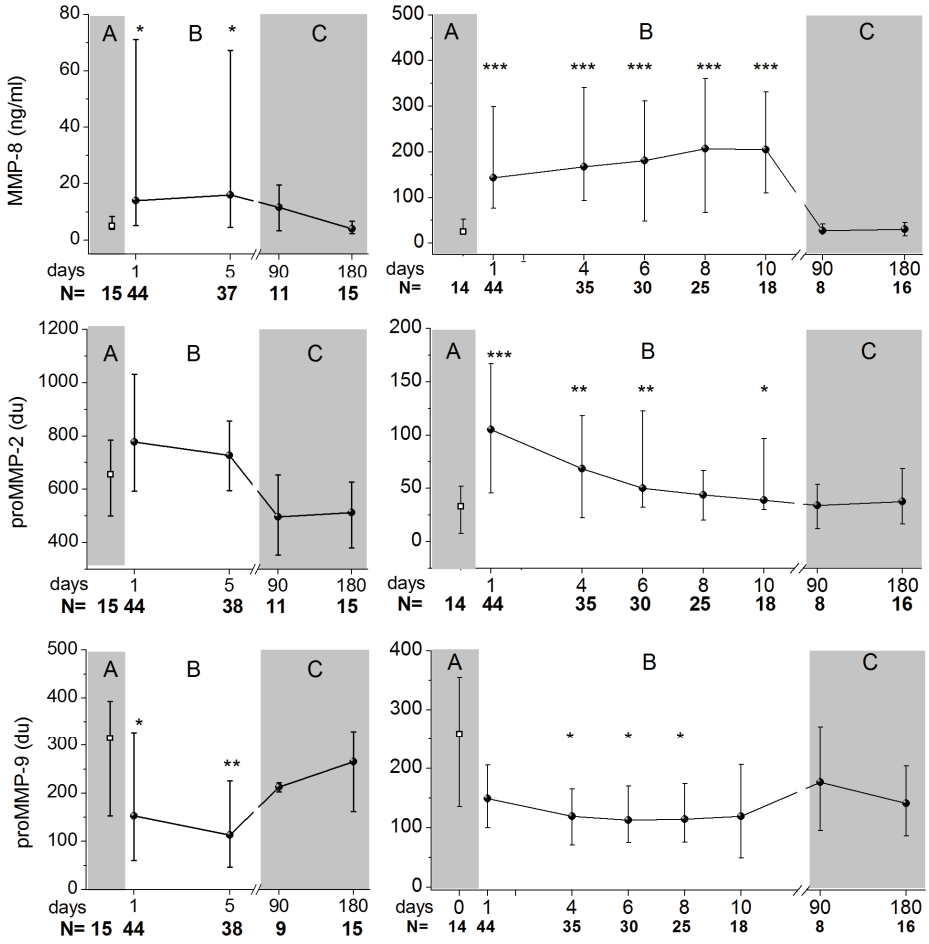

Fig. 21. MMP-8, proMMP-2 (62 kDa) and proMMP-9 (92 kDa) levels in patients with severe sepsis and in healthy controls. Results from the suction blister samples are on the left and from the serum samples on the right. Panel A presents the control value, panel B the values of all the patients in severe sepsis and panel C the values of the surviving patients at three and six months after severe sepsis. The diagonal lines mark the range from 25th to 75th percentile. Statistically significant differences between the control values and the values of the patients at each measuring point are marked with asterisks above the values of the patients (*= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$). The development of patient number (N) is expressed below each diagram.

6 Discussion

6.1 Microcirculatory response in intact abdominal skin in severe sepsis and in experimental wound model

In this study skin blood flow measured with laser Doppler flowmeter was increased on the fourth day in the septic patients in comparison with the controls both in the early blister induced four days earlier and in the freshly induced late blister. Also, in the second measurement of late blister blood flow was higher than in the controls.

With this method increased blood flow has been found also previously in small studies in forearms of sepsis patients (Young & Cameron 1995, Sair *et al.* 2001). However, the laser Doppler method does not provide information of the caliber of vessels perfused as Orthogonal polarisation spectral imagining (OPS), but measures the density and velocity of the red blood cells in the arterioles, capillaries and venules of the upper plexus of the dermis (Choi & Bennett 2003, Eun 1995, Groner *et al.* 1999). Thus, high flows determined by this method do not exclude microcirculatory maldistribution observed sublingually by OPS in sepsis (De Backer *et al.* 2002, Sakr *et al.* 2004). Indeed, it has been shown in experimental sepsis that LPS exposure dilates slightly the arterioles and venules, but decreases the functional capillary density by 50% on the first day (Hoffmann *et al.* 2004) and that the capillary density is low up till 3 days (Hoffmann *et al.* 1999). The reduction of capillary densities has also been noted in other organs in experimental models (Vincent & De Backer 2005). In our study the blood flow in skin was different from controls first on the fourth day of the study. This is in accordance with a previous case-series report with sepsis patient, in which the sepsis patients did not differ from the controls at 24 and 48 hours (Kubli *et al.* 2003).

All in all, even though the overall microcirculation from the beginning of the arterioles to the end of the venules seem to be normal or increased, the distribution of the flow is severely disturbed in the smallest vessels and failure to improve capillary flow is related to poor outcome (Sakr *et al.* 2004).

6.2 Balance of the ECM remodelling in skin is disturbed in clinical sepsis

Protein concentration and collagen synthesis in intact skin have not been studied in experimental nor clinical sepsis. However, studies on wound healing in sepsis are in accordance with ours: Collagen content was decreased and re-epithelisation delayed in incisional wounds of mice with sepsis induced by intra-abdominal infection. In addition, macrophage and neutrophil counts in the wound remote to infectious focus were decreased in comparison to the control mice on day 3 of healing (Rico *et al.* 2002). In humans Clark and colleagues have shown that the levels of hydroxyproline, a collagen-specific amino acid, are decreased by sepsis in major trauma (Clark *et al.* 2000).

This study revealed that protein concentration in blister fluid is lower in skin blister fluid derived from patients with severe sepsis than in the controls. Furthermore, the PIIINP/PINP ratio corresponds to that of granulation tissue. In normal skin PIIINP makes up approximately 10% of collagen, but in granulation tissue approximately 30% (Broughton *et al.* 2006). In addition, the marker of collagen I degradation, ICTP was systemically increased and collagen degrading matrix metalloproteinases MMP-8 and MMP-2 were increased in blister fluid. These results suggest that the balance of ECM remodelling in skin is severely disturbed in severe sepsis. This could be one explanation for the increased susceptibility to pressure ulcers of sepsis patients (Shahin *et al.* 2008) and could add to the pathology of the increased vascular permeability seen in sepsis. Interstitial protein composition of the skin is after all one of the regulators of capillary permeability. It has become clear in studying burn wound oedema that if the collagen coils and hyaluronate springs holding the ECM together are disrupted, the ability of the interstitium to withstand fluid accumulation markedly decreases. Additionally, as the osmotically active particle number increases, fluid accumulation further increases (Eckes *et al.* 2006).

6.3 ECM remodelling at the whole-body level

During this study I became fascinated with describing severe sepsis as a whole-body wound. It was Waydhas that first used this term in describing the elevated PIIINP values in trauma induced multiple organ failure (Waydhas *et al.* 1993). Just like during wound healing, coagulation, inflammation and tissue regeneration processes are activated with the difference that in severe sepsis this occurs at

whole-body level, and even in undamaged tissues causing dysfunction on different organs. The challenge is to distinguish adaptive response from maladaptive fibrogenetic response.

Traditionally fibrogenetic response in healing has considered to fill passively the gaps that the parenchymal injury left behind. However, evidence of the active dialog of plastic parenchymal cells and myofibroblasts is emerging. In my opinion, myofibroplasia and fibrosis could be seen as a safety net that supports the parenchyma as long as the reparation is ongoing. If the regeneration of the parenchyma is complete, the fibrosis can reverse, if not, it persists and creates a scar to a best-of-ability to repair. An fascinatingstudy by Fujigaki *et al.* (Fujigaki *et al.* 2005) supports this idea. They induced acute renal failure in rats with uranyl acetate. They showed histologically and with electron microscopic studies that after induction of acute renal failure, peritubular α-SMA positive myofibroblasts appeared and extended along the damaged proximal tubules and almost disappeared after recovery. Furthermore inhibiting cytoskeletal movement and myofibroblast differentiation resulted in more dilated proximal tubules, more severe renal dysfunction and inhibition of regenerative repair. This kind of theory would better fit to organ dysfuction in sepsis as they seem reversible.

The present studies showed that procollagen III propeptide levels and the PIIINP/PINP ratio are increased in patients with severe sepsis, but returned to normal by three and six months. During healing collagen III is deposited, whereas collagen I is laid down in later stages of healing (Broughton *et al.* 2006). Thus our results suggest that granulation tissue, like collagen profile, features the regenerative process in severe sepsis. ARDS is the only organ failure in severe sepsis in which fibrosis has been studied clinically. Type I and III procollagen propeptides have been shown to be increased in serum as well as in bronchoalveolar lavage fluid. Furthermore, fibroproliferation begins already on the first day of disease and is associated with increased risk of death (Clark *et al.* 1995, Meduri *et al.* 1998, Marshall *et al.* 2000b). Multiple organ failure derived from severe trauma is much alike as seen in severe sepsis, and Waydhas has shown that elevated PIIINP levels are associated with severity of organ failures and mortality (Waydhas *et al.* 1993). In our study PIIINP and PINP levels correlated with disease severity and mortality (II), but the small samples size limits from analysing PIIINP and PINP as prognostic markers. When interpreting these results it must be kept in mind that the response seen in serum is a summation of synthesis from different organs and that the response may vary. In

ARDS the synthesis is overt (Marshall *et al.* 2000b), but in uninjured skin it seems to be depressed (III).

6.4 Resolution of fibrosis and fibrosis restricting factors

Reversibility of fibrosis has been a debated issue in chronic fibrosis of parenchymal organs. Traditionally fibrosis has been considered irreversible, but evidence of regression of fibrosis is growing. In liver, regression of fibrosis follows the attenuation of chronic inflammation and decrease in TGF-β signalling (Kisseleva & Brenner 2006). However, cross-linking of fibrillar collagens restricts remodellation and is considered to be a structural change whereafter the fibrosis becomes irreversible (Iredale 2007). Studies on fibrosis in acute organ failures are few and the resolution of fibrosis after acute organ failure is even more scantly studied. Our results on reduced PIIINP and ICTP levels at 3 and 6 months in surviving patients imply that the remodellation response is activated in disease state and attenuates later on.

Furthermore, the same kind of counterbalance that prevails in coagulation-anticoagulation and inflammatory-anti-inflammatory in severe sepsis seems to feature the ECM remodelling. The marker of collagen I degradation was increased already on the first day of the study (II). In addition, the levels of matrix metalloproteinases -8 and -2 were elevated already in the beginning of the disease (IV). PINP/ICTP ratio was 1/5 of that in normal state indicating that collagen I degradation in particular by MMPs is enhanced, whereas PINP synthesis was quite unchanged (II).

6.5 Matrix metalloproteinases – multifunctional actors of inflammation and repair

MMPs have been previously studied in the beginning of human sepsis. Nakamura *et al.* were the first to report evidence of elevated MMP-9 levels in association with mortality in sepsis (Nakamura *et al.* 1998). Hoffmann *et al.*, demonstrated elevated plasma levels of MMP-9, TIMP-2, and TIMP-1 on the first day of severe sepsis and furthermore significantly higher TIMP-1 levels in non-surviving patients (Hoffmann *et al.* 2006). Furthermore, Lorente *et al.* reported elevated MMP-10 and TIMP-1 levels in the beginning of severe sepsis and suggest that these could be new biomarkers of disease severity and mortality in sepsis (Lorente *et al.* 2010). Also, in secondary peritonitis and consequent septic shock,

the MMP-8 levels in peritoneal fluid were shown to be increased in comparison with serum levels (Hastbacka *et al.* 2007).

Each of the present studies was conducted in the beginning of sepsis and thus our study is the first one providing the information on the evolution of the levels during severe sepsis. Originating from this basis it is not contradictory that our study found low MMP-9 levels in serum from the fourth day on. Taken together MMP-9 levels seem to be increased in early sepsis, but attenuate later on. That MMP-9 levels are low in blister fluid (IV) and epidermal wound healing is delayed (I) seem reasonable in the light of the evidence that MMP-9 seems to enable migration of epithelial cells by degrading collagen IV in dermoepidermal junction (Oikarinen *et al.* 1993). However, the reason for low MMP-9 levels remains to be elucidated.

MMP-2 has not been noted to be associated with septic organ failure in heart and lung (Torii *et al.* 1997, Wohlschlaeger *et al.* 2005). In addition it has been proposed to have roles in increasing vascular permeability, promoting neutrophils and regulating IL1-levels (Ito *et al.* 1996, McQuibban *et al.* 2002, Reijerkerk *et al.* 2006). Accordingly the MMP-2 levels were increased in serum more in the early phases of sepsis. Surprisingly MMP-2 expression was increased in blister fluid from uninjured skin and active form was found in all sepsis patients, but not in the controls (IV). Non-survivors and patients with more severe organ failures had higher MMP-2 levels in suction blister fluid than survivors and patients with less severe organ dysfunction, respectively.

MMP-8 levels were elevated during the whole study period in sepsis and in skin blister fluid, but no correlations to disease severity and mortality could be found. This can be a result of MMP-8 having both detrimental and beneficial roles in sepsis. In lung excessive fibrosis is associated with high mortality. MMP-8 degrades collagens I and III and MMP-8, which is bound to neutrophils is resistant to TIMP inhibition (Owen *et al.* 2004). In this way MMP-8 can function as a beneficial antifibrotic factor in lung. However, as all the MMPs are multifunctional, further studies are needed to map out exactly the different roles of MMPs in sepsis.

6.6 Strengths and limitations of the study

A study setting with critically ill sepsis patients causes challenges for standardisation and thus causes some limitations for the interpretation of the results. Firstly, the final sample size was small due to multiple reasons. As the

study was planned to be descriptive and ethical aspects were also taken into consideration, the planned sample size was decided to be 44 patients. Power analysis could not be conducted due to the lack of corresponding studies at the time of design. During the follow up, high mortality further decreased the sample size. Furthermore, high morbidity after intensive care unit stay decreased the number of patients able to participate in control measurements after 3 and 6 months. Thus, it is to be emphasised that the number of patients is too small for a reliable statement about the studied variables as prognostic markers of disease severity and outcome. However, many of the studied variables (PIIINP, PINP, ICTP, MMP-2) showed association to disease severity and/or mortality and can be studied further as prognostic markers. The decrease in patient number during the study causes some bias.

Secondly it was problematic to decide a control group that would be similar to the patient group in all aspects other than severe sepsis. The problem is that systemic inflammatory response can be activated also for reasons other than infectious insult, and is very common in ICU patients, especially in surgical ICUs (Brun-Buisson 2000). On the other hand, nearly all admission to ICU result from a need to support organ functions in acute or acute-on chronic organ failures in which collagen metabolism is potentially more or less affected (for instance, exacerbation of chronic heart failure or obstructive lung diseases). Then again in patients admitted, for example, due to intoxications, the elimination processes are affected, which means that this group is not an adequate control group either. Thus we considered it more reasonable to use healthy controls.

Thirdly, the blister wounds were induced without biochemical estimation of the level of inflammation. In sepsis the cytokine profile is individual and constantly changing, and up to date there are no reliable diagnostic batteries for revealing the phase of inflammatory response. Thus, all the suction blisters were induced within 48 hours of the appearance of the first organ dysfunction. The time window of 48 hours is long, but with the existing strict inclusion criteria, a shorter time frame would have markedly decreased the patients ability to participate.

Lastly, despite the strict inclusion criteria the majority of patients (61%) had chronic diseases which may affect collagen metabolism such as ischemic heart disease, chronic heart failure, diabetes and chronic obstructive lung diseases. Furthermore, the medications generally used in severe sepsis alter collagen metabolism; Vasopressor agents, hydrocortisone and APC have been shown to affect MMP-expression (Xue *et al.* 2004, Wohlschlaeger *et al.* 2005, Xue *et al.*

2007) and corticosteroids depress collagen deposition (Oikarinen *et al.* 1992b, Meduri *et al.* 1998). These confounding factors are practically impossible to eliminate from a clinical setting, but should be better analysed as confounding factors in forthcoming studies.

However, this study provides novel information of the healing response in sepsis. The study setting is interestingly in the middle ground of basic and clinical research and provides clinical evidence for the theories arising from experimental studies, e.g. from the field of translational medicine. Translational medicine is an innovative new field of medicine that focuses onapplying the knowledge of basic research to clinical implications and to improvements in public health. The research group composed of experts from multiple fields of clinical and basic medicine, made it possible to create an inventive study hypotheses that proved to be true and thus opened a new field in studying sepsis pathophysiology. Untill now when discussing the host response or healing response in severe sepsis the focus has been on coagulation and inflammation. This study widens the investigations to tissue healing processes possibly induced by the systemically activated coagulation and inflammation.

6.7 Ethical considerations

The sampling for the study was carried out in the beginning of a severe disease, which awakens some ethical concerns. The majority of the patients were unconscious or on mechanical ventilation at the time of the study admission and thus the consents were mainly obtained from the next of kin. When the patient was able to give his/her own consent, the study was discontinued if wished. The person giving the consent was informed orally and in writing. It was emphasized that a refusal wouldt not affect the care given, and that the study can be discontinued whenever so wanted.

The suction blister method is a relatively non-invasive, nearly pain free method for separating the epidermis from the dermis and for obtaining suction blister fluid that resembles closely skin interstitial fluid. There is a theoretical chance for blister wound infection especially with this patient group. The patient or next of kin was informed about this prior to giving consent. No infectious complications were seen during the sampling.

The study data was stored as required by data registration requirements kept in a safe place and no data including patient names or IDs have been sent through

e-mail. Only persons belonging to the study group and two study nurses were allowed to access the study records and the names of the study group was included in the study information given to the patient.

The Ethical Board of Oulu University Hospital had approved the research plan.

6.8 Methodological considerations

This study aimed at examining the effect of severe sepsis on some aspects of wound healing and connective tissue. However, patients with severe sepsis are a very heterogeneous population with chronic diseases in the background which affect connective tissue metabolism. In order to minimize the effect of these other conditions, a list of exclusion criteria had to be applied. This helps to distinguish the effect of sepsis, but inevitably leads to restrictions in the generalisation of the results. These results thus apply to adult population with none of the chronic diseases and other conditions mentioned in the exclusion criteria (I-IV). Other factors that reduce the generalisability of the results include the limited sample size and the fact that this was a one center-study.

The induction of suction blisters and the physiological bed-side measurements of transepidermal water loss and blood flow were carried out by MK and FG to avoid researcher dependent variability. The physical environment with air humidity and temperature as well as patient temperature and drug therapies were difficult to standardise in intensive care unit setting. However, in the measurement of water loss a closed chamber system was used to minimise the effect of external or body induced air flows. Furthermore, the values measured did not correlate with patient temperature, fluid balance or noradrenalin dose.

The laboratory methods for determining procollagen propeptides in serum and suction blister fluid have been standardised and reliable, and the measurements were carried out by an experienced laboratory technician. Similarly, experts were trusted in carrying out the measurements of matrix metalloproteinases. The gelatinases (MMP-2 and MMP-9) were measured with gelatine zymography that detects gelatin degrading MMPs according to their molecular weight.

6.9 Clinical implications and future perspectives

The findings of disturbed collagen turnover and delayed re-epithelisation are important in clinical reality as patients with severe sepsis often require invasive monitoring and surgical interventions. It is worthwhile to acknowledge that the healing capacity is not normal in sepsis. This is seen for instance in the higher susceptility to pressure ulcers (Shahin *et al.* 2008). Further research into the balance of interstitial proteins might help to create ways to enhance wound healing.

At the whole-body level the fibrogenetic response seems to vary between organs and it is not easy to distinguish between adaptive and pathological responses. In sepsis, organ failures mostly heal without permanent scars and thus mitochondrial pathology is suggested (Protti & Singer 2006). However, near to complete healing does not exclude transitient ECM remodelling as a feature of pathogenesis of septic organ failures (Weber 1997, Fujigaki *et al.* 2005). The potential regeneration supportive role of myofibroblasts and ECM should be further studied in acute organ failures in sepsis. These studies could in future provide opportunities to modulate the supportive role of ECM in sepsis. If the theoretical framework proved to be true, the antifibrotic strategies like corticosteroids and angiotensin II antagonizing could be investigated. (Weber 1997). Namely, despite of its role in blood pressure and fluid homeostasis control, renin-angiotensin system is shown to induce fibrinogenesis in heart, kidney, liver and lung (Lambert *et al.* 2010) In lung it has been shown that mice deficient of ACE-1 regulating ACE-2 displayed more severe ARDS, an effect attenuated by AT1 receptor antagonism (Imai *et al.* 2005). The mechanism of lung fibrosis is suggested to be that angiotensin II increases expression of TGF-beta and collagen mRNA expression in lung fibroblasts (Marshall *et al.* 2000b).

In addition to further examining the role of ECM in the pathophysiology of septic organ failures, the PIIINP, PINP, ICTP and MMPs studied here can be further studied as prognostic biomarkers of disease severity and mortality in sepsis. In that case they would be most plausible to examine as part of a larger panel with other promising severe sepsis biomarkers such as cytokines, C-reactive protein and procalcitonin (Pierrakos & Vincent 2010).

7 Conclusions

This study provides novel information on healing responses in severe sepsis in humans.

1. Serum procollagen propeptides I and III describing the net collagen I and III synthesis in the body have previously been studied in critical illness in acute lung failure and severe trauma. This is the first study that shows that the PIIINP levels are increased in severe sepsis, whereas PINP levels are not, making up a pronounced PIIINP/PINP ratio. This implies that the balance of collagen production resembles that of granulation tissue. Additively this is the first study to show that collagen I degradation measured with ICTP assay is increased in severe sepsis and that PINP/ICTP ratio is lower. Furthermore PIIINP and PINP levels correlated with disease severity and mortality.
2. PIIINP levels are increased in severe sepsis, whereas PINP levels are not, making up a pronounced PIIINP/PINP ratio. This implies that the balance of collagen production resembles that of the granulation tissue. In addition, this is the first study to show that collagen I degradation measured with ICTP assay is increased in severe sepsis, and that the PINP/ICTP ratio is lower. PIIINP and PINP levels correlated with disease severity and mortality
3. It was revealed for the first time that a crucial part of wound healing, the restoration of the epidermal barrier, is delayed. Additionally, the microcirculatory response to wounding seems to be pronounced in abdominal skin in severe sepsis. Furthermore, the microcirculatory flow measured with laser-Doppler is pronounced even in intact abdominal skin.
4. Thirdly, it was shown that the overall protein concentration and PINP and PIIINP levels are down regulated in suction blister fluid, which implies that the balance of the extracellular matrix consistence is disturbed in intact skin in sepsis. Then again in survivors the levels of PINP and PIIINP were up regulated at three months but returned to normal by six months.
5. During this study, arising interest evolved in examining the role of matrix metalloproteinases in severe sepsis. Our study supports the previous findings of elevated serum MMP-8 levels in sepsis and provides a longer time window to examine the fluctuation of the levels in time. Elevated MMP-8 levels provide one explanation for enhanced collagen I degradation. MMP-9 levels in serum and skin blister fluid were lower during the studied ten days contrary to previous findings of high levels in early sepsis. MMP-2 level has

not been previously studied in severe sepsis. This study provides novel information on the relevance of this MMP. The MMP-2 levels were found to be increased both in serum and in skin blister fluid and were associated with disease severity and mortality. Additionally, active MMP-2 was found in the suction blister fluid samples of all patients, but not in the control samples.

In conclusion, extracellular matrix remodelling is activated systemically and in intact skin in severe sepsis and may possess an important continuum of coagulation and inflammatory responses in severe sepsis. In addition, the epidermal healing of the experimental suction blister wound is delayed. At systemic level the synthesis of collagen III seems to be increased, whereas collagen I production is not altered in the same extent. Moreover, the propeptide levels are associated with disease severity and mortality and can be further studied as biomarkers of severe sepsis. The marker of collagen I degradation, ICTP is increased and high levels are associated with more severe disease. MMP-2 and MMP-8 serum levels are increased during sepsis, whereas MMP-9 shows a downward evolution. MMP-2 and MMP-8 expressions are increased and MMP-9 levels suppressed in the suction blister fluid from intact skin. All in all extracellular matrix remodelling occurs in sepsis with organ failures and it may posses an important continuum of coagulation and inflammatory responses in sepsis. An adequate fibrogenetic response seems to be crucial to successful healing as the markers of extracellular matrix remodelling correlate with disease severity and mortality.

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Original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Gäddnäs F, Koskela M, Koivukangas V, Risteli J, Oikarinen A, Laurila J, Saarnio J & Ala-Kokko T (2009) Markers of collagen synthesis and degradation are increased in serum in severe sepsis: a longitudinal study of 44 patients. Critical Care 13: R53.

II Koskela M, Gäddnäs F, Ala-Kokko T, Laurila J, Saarnio J, Oikarinen A & Koivukangas V (2009) Epidermal wound healing in severe sepsis and septic shock in humans. Critical Care 13: R100.

III Gäddnäs F, Koskela M, Koivukangas V, Laurila J, Saarnio J, Risteli J, Oikarinen A & Ala-Kokko T (in press) Skin collagen synthesis is depressed in patients with severe sepsis. Anestesia and analgesia.

IV Gäddnäs F, Sutinen M, Koskela M, Tervahartiala T, Sorsa T, Salo T, Laurila J, Koivukangas V, Ala-Kokko T & Oikarinen A (2010). Matrix metalloproteinases 2, 8 and 9 in serum and skin blister fluid patients with severe sepsis. Critical Care 14: R49.

Original publications are not included in the electronic version of the dissertation.

1047. Railo, Antti (2010) Wnt-11 signalling, its role in cardiogenesis and identification of Wnt/β-catenin pathway target genes

1048. Ahvenjärvi, Lauri (2010) Computed tomography in diagnostics and treatment decisions concerning multiple trauma and critically ill patients

1049. Haapea, Marianne (2010) Non-response and information bias in population-based psychiatric research. The Northern Finland 1966 Birth Cohort study

1050. Poikonen, Kari (2010) Susceptibility of human macrophages to *Chlamydia pneumoniae* infection *in vitro*

1051. Ojala, Kati (2010) Modern methods in the prevention and management of complications in labor

1052. Auvinen, Juha (2010) Neck, shoulder, and low back pain in adolescence

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