

Chapter 1

General Introduction & Outline of the Thesis

GENERAL INTRODUCTION

Despite decades of research, mortality and morbidity rates of sepsis and acute respiratory distress syndrome (ARDS) remain appallingly high. In the United States, an estimated 200,000 people die of sepsis each year [1]. Intrinsic factors contributing to the severity of these syndromes include severe biological or chemical threats, inappropriate immune responses, and generalized tissue damage. These factors contribute to the systemic inflammation, the generalized or localized vascular leak and the organ dysfunction that characterize sepsis and ARDS. Extrinsically, a direct therapy targeting and inhibiting underlying pathophysiological processes is still lacking. Recent insights stress the importance of endothelial barrier dysfunction and vascular leak for development of sepsis [2,3] and ARDS [4]. Taking the endothelium as starting point of this thesis, we searched for targets to improve both treatment and diagnosis of vascular leak during sepsis and ARDS.

In the first chapter of this thesis, we describe the endothelial barrier as fundamental determinant in the pathophysiology of ARDS and sepsis. Starting with the structure and function of the endothelium, we illustrate how dysfunction of the endothelial barrier leads to vascular leak. Subsequently, the phenomenon of vascular leak is put in the context of the physiology of fluid homeostasis, showing how endothelial barrier dysfunction and vascular leak contribute to edema formation. A description of sepsis and ARDS illustrates the relevance of endothelial barrier regulation and dysfunction for the clinical context. Throughout this chapter the unknown areas in the field of endothelial barrier regulation are highlighted, with a specific focus on how resolution of these issues may contribute to treatment and diagnosis of vascular leak in a clinical setting. This chapter ends with an overview of the work performed in this thesis.

Structure and function of the endothelium

The endothelium as anatomic structure was first described in 1865 by the Swiss anatomist William His, who described the endothelium as cell layer covering body cavities [5,6]. Although Hiss' original definition included blood vessels, lymphatic vessels and mesothelium-lined cavities, the endothelium is nowadays defined as the inner cell layer that covers blood and lymphatic vessels. Covering the whole vascular tree from large conductance vessels like the aorta and the vena cava to the smallest exchange vessels, the capillaries, the endothelial layer has an estimated surface of 271-350m² [7,8]. The thickness of this layer varies from 0.1µm (capillary) to 1µm (aorta) depending on the anatomical location.

Morphology

From a morphological point of view, the endothelium is a monolayer of tightly connected endothelial cells. Endothelial cells arise from the mesoderm shortly after implantation. This process starts with early formation of blood islands in the yolk sac, in which angioblasts form the precursors of endothelial cells [9]. Although endothelial cell shape varies between vascular beds [5], the general appearance of endothelial cells can be described as flat and stretched. The shape is determined by the interplay of endothelial cells with the extracellular environment [10]. At the luminal side the endothelium faces the content of the vascular lumen (plasma, solutes, platelets, red and white blood cells), at the basolateral side endothelial cells are attached to the extracellular matrix (basal membrane or interstitium) by focal adhesions, and at the lateral edges endothelial cells are connected to neighbouring cells by cell-cell junctions. Maturation of cell-cell junctions forms the ultimate step in the development of an endothelial monolayer, as junction maturation is followed by inhibition of cell division and spreading, a process called 'contact-inhibition' [11]. As a consequence of this contact-inhibition, growth or movement into the third dimension is inhibited or limited to pathological conditions [12,13], and endothelial cells are organized in a monolayer. Within this monolayer the shape of endothelial cells is largely determined by extracellular factors, in particular composition of the extracellular matrix [14,15], but also direction of the blood flow, in which endothelial cells tend to align [16,17]. The overall result is a continuous monolayer of endothelial cells that separate body fluids from body tissues and thus allows effective circulation.

Functions of the endothelium

The endothelium harbors a large repertoire of regulating functions, including regulation of hemostasis, vascular tone, leucocyte trafficking [18], innate immunity and metabolism [5,9]. The morphological structure of the endothelial monolayer illustrates the other major function of the endothelium, i.e. its function as a barrier. The tight structure of spread cells with mature interendothelial junctions precludes unrestricted passage of water, solutes and proteins from the circulation to the surrounding tissues. This barrier should, however, be considered dynamic rather than static [19,20]. For effective delivery of nutrients and oxygen to peripheral tissue, the endothelial barrier should on the one hand prevent leakage in conductance vessels to maintain perfusion pressure, and on the other hand allow for exchange in the capillaries and postcapillary venules. During exchange the endothelial barrier should allow unrestricted uptake of nutrients and proteins, but limit extravasation of water to prevent edema formation. In addition, at certain moments the endothelial barrier should allow passage of immune cells to counteract microbial threats, while in healthy conditions it has to inhibit extravasation of immune cells to prevent

undirected tissue damage. Altogether these conditions require a highly dynamic endothelial barrier, the permeability of which is tightly regulated in space and time.

Endothelial barrier regulation

Plasma constituents may pass the endothelial barrier through endothelial cells (transcellular transport) or through clefts between endothelial cells (paracellular transport) (Figure 1). Under basal conditions larger molecules (>3 nm molecular radius, like albumin) pass the barrier via transcellular transport (in endosomes and vesiculo-vacuolar organs), free water passes via aquaporins, while smaller solutes (<3 nm molecular radius) and immune cells may pass the barrier via intercellular spaces [21]. As transcellular transport is a constitutive process, the size of the intercellular space is the main determinant of endothelial barrier function. Changes in endothelial cell shape and cell junction integrity in turn determine intercellular space and paracellular transport.

Three protein complexes (all belonging to or in conjunction with the endothelial cytoskeleton) contribute to a closed configuration of the intercellular space in a resting, mature monolayer: 1) cell-cell junctions, 2) a cortical F-actin band, and 3) cell-matrix adhesions (Figure 1, insert). Several types of junctions have been described to connect endothelial cells – gap junctions, tight junctions and adherens junctions (AJ). The main function of gap junctions is intercellular communication [22]. Tight junctions may play a role in endothelial barrier regulation via the transmembrane proteins claudin and occludin [23], but AJ have been studied most extensively in this context [21]. The core protein of the AJ is VE-cadherin, a transmembrane protein that forms a calcium-dependent homodimeric bond with VE-cadherin of adjacent endothelial cells [24]. The intracellular domain of VE-cadherin is connected to the actin cytoskeleton via α -catenin and β -catenin [25]. In addition to this core complex the AJ contains regulatory proteins like kinases, phosphatases and RhoGTPases, which together regulate AJ integrity [26]. In a healthy monolayer, the actin cytoskeleton is mainly localized in the peripheral parts of the cell. This cortical F-actin band is thought to support AJ integrity [27], and to enhance the strength of cell-cell contacts [28]. Besides contact with adjacent endothelial cells, endothelial cells attach to the extracellular matrix via cell-matrix adhesions. Core proteins for cell-matrix adhesion are integrins, transmembrane proteins that connect the endothelial cytoskeleton with the extracellular matrix [29,30]. The integrin extracellular domain binds the fibrils of the extracellular matrix, while their intracellular domain is connected to the actin cytoskeleton via a large regulatory protein complex, the focal adhesion complex [31]. Cell-matrix adhesions contribute to the spread morphology of endothelial cells [14], and have been shown to support cell-cell contacts, either directly by presence in cell-cell contacts [32] or indirectly via integrin-mediated signaling [33,34]. Although this may contribute

to integrity of the endothelial barrier, the exact role of cell-matrix interactions in endothelial barrier regulation remain poorly understood.

Together, functional cell-cell contacts, a well-organized cortical F-actin band and firm adhesion of endothelial cells to the extracellular matrix limit the intercellular space. The limited size of the interendothelial space resulting from these mechanisms precludes passage of larger molecules via the paracellular pathway under basal conditions. Widening of the interendothelial cleft and the formation of interendothelial gaps leads to the water and solute extravasation as observed in inflammatory conditions.

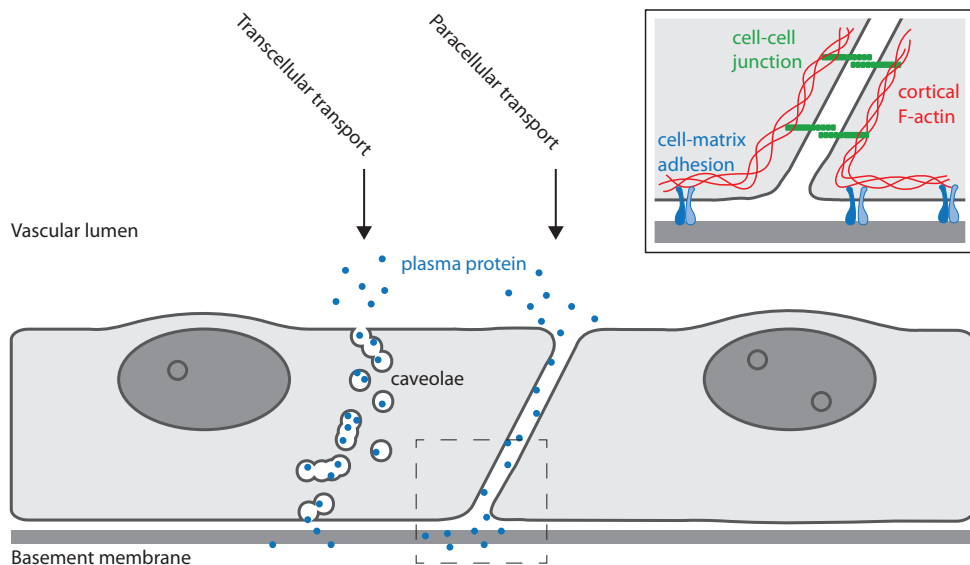


Figure 1 – Modes of protein transport through the endothelial layer. Transport of plasma constituents over the endothelial barrier through endothelial cells by transcellular transport and between endothelial cells by paracellular transport. Insert: Actin cytoskeleton in conjunction with the main endothelial adhesion complexes (cell-matrix adhesions and cell-cell junctions).

Endothelial barrier dysfunction

Dysfunction of the endothelial barrier is characterized by formation of large gaps between the endothelial cells and disruption of the endothelial monolayer. These changes in monolayer morphology follow structural changes in the endothelial cytoskeleton: breakdown of endothelial cell-cell contacts, contraction of endothelial cells due to increased centripetal actomyosin contractility, and partial detachment of endothelial cells from the extracellular matrix. Together these changes result in rounding up of cells and formation of interendothelial gaps.

Signaling events during endothelial barrier dysfunction

The changes of the endothelial cytoskeleton leading to gap formation are the precipitate of a cascade of biochemical events inside the endothelial cell. Inflammatory mediators and growth factors bind and activate receptors on the endothelial surface. Inside the cell the activated receptors initiate a number of signaling pathways. Influx of calcium and activation of the RhoA/Rho kinase/Myosin Light Chain pathway induce gross changes in actin morphology – the cortical actin band disappears and is replaced by contractile actin fibers [35,36], which exert centripetal contractile forces [37]. The activation of a number of tyrosine kinases (amongst others Src) leads to phosphorylation of AJ proteins and destabilisation of cell-cell contacts [38,39]. In parallel, inactivation of the RhoGTPase Rac1 reduces AJ stability and reduces the number of cell-matrix adhesions at the cell periphery [40]. Redistribution of cell-matrix adhesions may also result from activation of tyrosine kinases. In fibroblast studies, the Abl family of tyrosine kinases (c-Abl and Abl-related gene) has been shown to regulate the distribution of cell-matrix adhesions [41,42]. However, the contribution of cell-matrix adhesion distribution and particularly the role of Abl kinases in endothelial barrier disruption remain unclear. Finally, during endothelial stimulation there is activation of kinases that are not known to be involved in endothelial barrier regulation. AMP-activated Kinase (AMPK) is an example of such a kinase. AMPK is a serine/threonine kinase that is activated upon endothelial stimulation [43]. It is known to be involved in cellular energy metabolism [44], but the link to barrier regulation remains poorly understood.

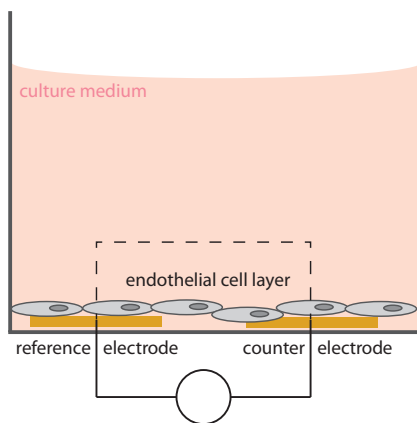
Conditions associated with endothelial barrier dysfunction

Although the structural and biochemical changes described above are not limited to inflammation alone, inflammatory mediators play a role in virtually all hyperpermeability states. Immune reactions elicited by neutrophils and macrophages lead to release of circulating factors like interleukins (Il-1 β , Il-6, Il-8), histamine and growth factors (Vascular Endothelial Growth Factor, VEGF). During destruction of bacteria by the immune system, parts of the bacterial wall particles like lipopolysaccharide (LPS) are released [45]. Tissue damage and thrombocyte disposition give rise to locally enhanced concentrations of thrombin [46,47]. All these factors bind and activate endothelial cells. Endothelial activation in turn results in endothelial barrier disruption via signaling cascades, and in the release of additional barrier-disruptive agents (like angiopoietin-2). In patients with sepsis and/or ARDS the plasma concentrations of these circulating factors correlate well with vascular leak, edema formation [48,49] and mortality [50,51]. Thus, inflammatory mediators disrupt the endothelial monolayer via breakdown of cell-cell contacts, the detachment of cell-matrix adhesions and the actomyosin contraction.

Measuring endothelial barrier dysfunction

The widening of the intercellular space and the formation of intercellular gaps spur the paracellular extravasation of water and plasma constituents (vascular leak). Barrier dysfunction can be measured in cultured endothelial cells, with systems like Electrical Cell Substrate Impedance Sensing (ECIS), which measures the electrical impedance of a cultured endothelial monolayer (Figure 2) [52], or with a Transwell™ assay, which measures the passage of a tracer molecule over an endothelial monolayer grown on top of a microporous filter (Figure 2) [53]. *In vivo*, vascular leak can be visualized with the Miles assay, in which a dye (e.g. Evans Blue) is injected in the circulation of an animal. Intradermal injection of barrier-disruptive agents like VEGF leads to local extravasation of the dye, which in turn can be quantified by photospectrometrical analysis of the skin [54]. The intensity and duration of endothelial barrier dysfunction depends on the barrier-disruptive agent responsible for the breakdown (histamine – minutes, thrombin – hours, TNF α – hour to days) and the functionality of intrinsic endothelial recovery mechanisms [21].

Electrical Resistance Measurement



Macromolecule Passage

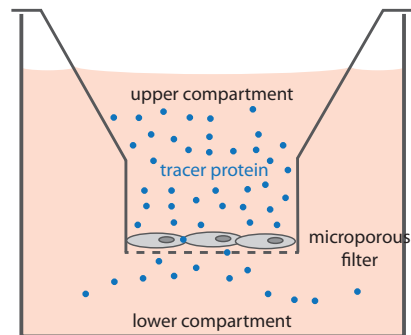


Figure 2 – Assays for measurement of endothelial barrier function and vascular leak. A) Left: Electrical resistance measurement of endothelial barrier function with Electrical Cell-substrate Impedance Sensing (ECIS®). Endothelial cells are grown on top of two gold electrodes. Resistance is measured before and after treatment of cells with drugs or vaso-active agents. Right: Macromolecule passage measurement using Transwell microporous filters. Endothelial cells are grown on top of the filter. A tracer molecule together with vaso-active agents are added to the upper compartment. Macromolecule passage is then measured by regular sampling of the lower compartment.

Endothelial barrier dysfunction & Edema formation

Ultimately endothelial barrier dysfunction leads to vascular leak and fluid accumulation in tissues and organs (edema). Yet, besides endothelial barrier dysfunction, edema formation depends on other parameters and safety mechanisms. The relation between endothelial barrier function and fluid extravasation or vascular leak is described in the Starling equation. The relation between vascular leak and edema formation depends among others on the balance between fluid extravasation and lymphatic clearance.

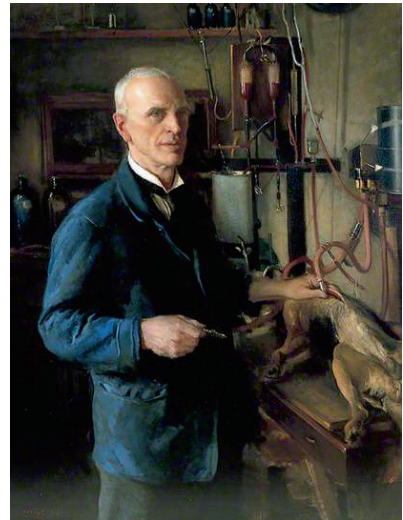


Figure 3 – Ernest H. Starling. Painting of Ernest H. Starling, an English physiologist who first described the relation between fluid passage, hydrostatic pressure and oncotic pressure. Most of his famous experiments on the absorption of fluids [55] were performed in dogs. Painting by Walter Westley Russell (1926), UCL Art Museum Collections.

The Starling equation and its revision

Whether or not endothelial barrier dysfunction leads to fluid extravasation, depends among others on difference in hydrostatic pressure and oncotic pressure between the vascular lumen and the interstitial space. This relation is described in the Starling equation, formulated in 1896 by the British physiologist Ernest Starling (Figure 3) [55]:

$$J_v = K_{fc} * ([P_i - P_e] - \sigma * [\pi_c - \pi_i])$$

In this equation, J_v is the net fluid passage over the endothelial monolayer, K_f the filtration coefficient, P the hydrostatic pressure of the vascular lumen (P_i) and the interstitium (P_e), while π is the oncotic pressure of the vascular lumen (π_i) and the interstitium (π_e). The σ is the reflection coefficient, which corrects for the effectiveness of the oncotic pressure difference in the net driving force. The functionality of the endothelial barrier affects fluid passage at two levels in

the Starling equation: 1) by changing K_{fc} , which is the product of the hydraulic conductivity and the surface area of the endothelium. Dysfunction of the endothelial barrier (at an assumed fixed endothelial surface area) enhances K_{fc} and thus net fluid passage (vascular leak). 2) In case of severe endothelial barrier dysfunction, proteins can freely pass from the capillary to the interstitial space (σ approaches 0), rendering any oncotic pressure difference ineffective. Although *in vivo* excess amounts of protein are quickly cleared from the interstitium and differences in $[\pi_i - \pi_i]$ are maintained (unless at very low plasma levels $\text{Alb} < 10\text{g/l}$) [56], the protective effect of oncotic pressure difference on vascular leak falls away during endothelial barrier dysfunction [57]. For conditions in which the endothelial barrier dysfunction is severely impaired (like sepsis and ARDS) and constant endothelial surface area is assumed, vascular leakage relates to endothelial barrier dysfunction as according to the following simplified Starling equation: Vascular leak = Endothelial barrier function * hydrostatic pressure difference. This relation, as applied to vascular leakage in the lungs, is illustrated in Figure 4A [57].

Late experiments, in which all four forces were measured simultaneously, have questioned the accuracy of the Starling equation at several levels [58]. First, according to the classical Starling equation, net fluid filtration takes place in the arterioles (due to high intravascular hydrostatic pressure, P_i), which leads to an increase in intravascular protein concentration. This elevation of the intravascular oncotic pressure (π_i) results in net fluid absorption from the interstitium to the vascular lumen of the venules (in which $P_i < \pi_i$). However, *in vivo* measurements have shown that fluid absorption with few exceptions does not occur in the body [58]. Second, other experiments have shown that the P_i is virtually always higher than the π_i , even in venules [59]. Third, the interstitial protein concentration (π_i) influences J_v to a lesser extent than expected on the basis of the classical Starling's principle [60]. One important explanation for this supposed inaccuracy of the Starling equation is the glycocalyx, a thin layer of proteoglycans that covers the endothelium at the luminal side [61]. It covers both endothelial cell bodies and the intercellular spaces, and restricts passage of larger molecules. The 'filter' property of the glycocalyx is responsible for a very low protein concentration in the intercellular space just below the glycocalyx. Therefore, the true oncotic pressure difference results from the protein concentration in the vascular lumen minus the protein concentration in the subglycocalyx intercellular space [58]. Together, this revision has yielded the following insights: a) The intravascular pressure is substantially higher than originally assumed by Starling, b) due to the glycocalyx the osmotic pressure gradient is always high and directed inwards, c) this osmotic pressure gradient always opposes filtration, but (almost) never reverses filtration, and d) interstitial protein concentration hardly influences fluid filtration. Several implications for the pathophysiology of vascular leak and edema arise here [62]. First, the endothelial glycocalyx is an important determinant of the osmotic pressure gradient that

opposes filtration. Therefore, damage to the glycocalyx, as observed in inflammatory conditions, may contribute to vascular leak and edema formation [61]. Second, as absorption hardly occurs, clearance of the interstitium from excess fluids almost fully depends on lymphatic clearance [63].

Lymphatic clearance

Whether vascular leak results in fluid accumulation and edema formation depends on lymphatic clearance, which provides a safety mechanism against edema formation. An increase in interstitial fluid leads to a rapid enhancement of lymphatic flow (Figure 4B). In a classic review on pulmonary edema, Staub demonstrates that pulmonary lymphatic flow may increase up to 13-fold in cases of sepsis (from 4mL/h at baseline to 52mL/h after induction of *Pseudomonas* bacteremia). Edema develops only when lymphatic capacity is reached and vascular leak overrides lymphatic flow [63,64].

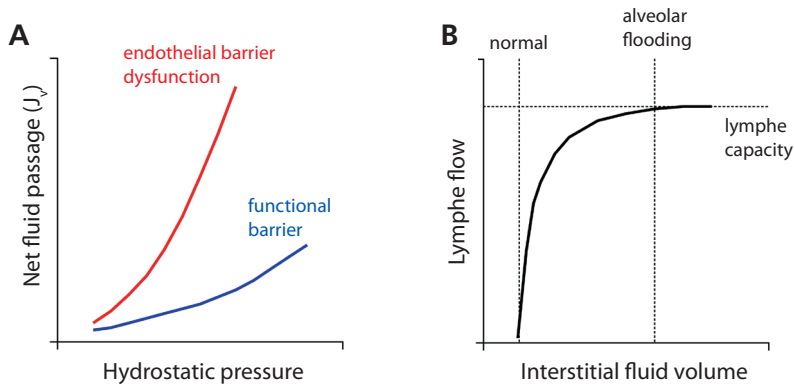


Figure 4 – Physiological factors influencing vascular leak and edema formation. A) The effect of endothelial barrier function on the relation between hydrostatic pressure and net fluid passage. Figure adapted from [57]. B) The preventive effect of pulmonary lymphic drainage on edema formation. Interstitial fluid volume may increase up to several times before alveolar flooding occurs. Figure adapted from [64].

If mentioned safety mechanisms are insufficient, edema develops as utter consequence of endothelial barrier dysfunction. The edema formation itself explains part of the high mortality and morbidity observed in syndromes characterized by endothelial barrier dysfunction. Interstitial fluid accumulation enhances interstitial pressure. This enhanced interstitial pressure combined with the loss of intravascular pressure (due to shock) results in loss of perfusion, hypoxia, ischemia and finally organ damage and dysfunction [65]. In addition, fluid accumulation may directly impair organ function when fluid interferes with organ function. Alveolar flooding as observed in pulmonary edema severely impairs gas exchange by enhancing the length of the diffusion distance from alveolar space to the capillary lumen and vice versa [4].

Syndromes characterized by endothelial barrier dysfunction and vascular leak

Evidence at several levels has stressed the contribution of endothelial barrier dysfunction to the pathophysiology of sepsis and ARDS. In animal models inhibition of endothelial barrier disruption strongly reduces mortality from sepsis or ARDS [66-68], whereas impairment of barrier-protective mechanisms has a deleterious effect on sepsis phenotype [69-70]. In clinical studies, endothelial barrier dysfunction directly relates to and predicts mortality of sepsis and ARDS, independent of comorbidity [71,72]. Together sepsis and ARDS form the leading cause of mortality in the ICU population.

Sepsis

Sepsis is a systemic illness caused by microbial invasion of normally sterile parts of the body [73] or the presence of infection presenting with systemic manifestations of an infection [74]. Sepsis is diagnosed in the presence of a number of non-specific inflammatory responses (SIRS criteria) with evidence for infection. The Systemic Inflammatory Response Syndrome (SIRS) criteria comprise: body temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$, heart rate $>90/\text{min}$, respiratory rate $>20/\text{min}$ or $\text{P}_a\text{CO}_2 <32\text{mmHg}$, and a white blood cell count $>12 \times 10^6/\text{mL}$ or $<4 \times 10^6/\text{mL}$ [75]. 'Severe sepsis' is defined as sepsis together with evidence for hypoperfusion or failure of at least one organ system, while 'septic shock' refers to sepsis accompanied by hypotension (systolic pressure $<90\text{mmHg}$ or mean arterial pressure $<65\text{mmHg}$), despite fluid resuscitation and/or use of vasopressors [75]. Central in the development of sepsis – and distinguishing sepsis from other infections – is a damaging host response to infection. This host response is characterized by systemic release of numerous cytokines ('*cytokine storm*'). These cytokines in turn invoke the processes like coagulation, tissue damage and vascular leakage that are responsible for the development of systemic inflammation [76,77] and systemic vascular leak [78].

In the United States sepsis has an estimated incidence of 3 per 1000 inhabitants each year, leading to 750,000 new cases each year. With mortality rates ranging from 25-30% (severe sepsis) up to 40-70% (septic shock) [73], sepsis has annual death rates of 200,000 in the United States [1]. In the Netherlands sepsis has a reported incidence of 0.60 per 1000 inhabitants per year. Annually 9700 patients with sepsis, 8600 with severe sepsis and 3900 patients with septic shock are admitted to the ICU [79]. Worldwide 15-19 million cases of sepsis are estimated to occur each year [80].

Acute Respiratory Distress Syndrome (ARDS)

ARDS is a collection of clinical symptoms which together lead to severe and acute respiratory failure. Hallmarks of ARDS are pulmonary edema and impaired gas exchange as evidenced by

low arterial oxygen and high carbon dioxide. This syndrome may result from physical, chemical or infectious hazards, including high tidal volume ventilation, drowning, radiation, pancreatitis, acid aspiration, chemotherapy (e.g. bleomycin), blood transfusion and infection (sepsis or pneumonia). For long, ARDS was diagnosed according to the 1994 American European Consensus Conference (AECC) criteria, which included 1) acute onset, 2) arterial hypoxemia with a P_aO_2/FiO_2 ratio <200 or 300 , 3) bilateral densities on chest X-ray and 4) absence of left atrial hypertension to exclude cardiac pulmonary edema [81]. Recently clinical criteria have been revised, yielding the Berlin criteria [82]. According to the Berlin criteria ARDS is defined by the presence of acute onset of symptoms, bilateral opacities on chest imaging (not explained by effusion, collapse or nodules) and pulmonary edema (not fully explained by cardiac failure or fluid overload), and classified by the degree of hypoxemia (mild P_aO_2/FiO_2 200-300mmHg, moderate P_aO_2/FiO_2 100-200mmHg, severe P_aO_2/FiO_2 <100 mmHg) [82]. Central concepts in the pathophysiology of ARDS are dysregulated inflammation, lung tissue damage and loss of the alveolocapillary barrier [4]. The alveolar flooding that follows breakdown of both endothelial and alveolar epithelial monolayers well explains the excessive edema formation observed in ARDS patients. Methods to assess vascular leak and edema formation in critically ill patients include the pulmonary leak index (PLI) which measures the amount of radiolabeled plasma protein inside the thorax, but outside the pulmonary circulation [83], and the extravascular lung water (EVLW), which measures the amount of extravascular water in the lung tissue by single or double indicator dilution techniques [84]. Epidemiological data of ARDS may vary according to definition, study population and geographic region. A recent study reported an incidence of 38.9 per 100,000 person-years in the United States [85]. A large Scandinavian trial reported an incidence of 31.4 (17.9 for ALI and 13.5 for ARDS) per 100,000 per year [86], while Dutch statistics reported an incidence of 53.3 (29.3 for ALI and 24.0 for ARDS) per 100,000 per year [87]. Like incidence, mortality rates vary between studies. Yet, a series of clinical trials from the NHLBI ARDS Clinical Trials Network suggest a reduction in mortality from 36% in 1998 to 22% in 2008 [88]. This reduction in mortality may be contributed to the implementation of effective interventions like low-tidal volume ventilation and conservative fluid resuscitation [4].

Other syndromes/diseases associated with endothelial barrier dysfunction

In addition to sepsis and ARDS various diseases result to some extent from endothelial barrier dysfunction. Systemic Capillary Leak Syndrome is defined as systemic loss of endothelial barrier function, leading to profound hypotension (shock) and generalized edema [89]. Although the pathophysiology of this disease has not been clarified, recent publications point toward a mediator role of inflammatory cytokines like VEGF and angiotensin-2 [90]. Endothelial barrier disruption

takes place at large scale in conditions like anaphylactic shock [91] and C1-esterase inhibitor deficiency [92]. At a microscopic scale endothelial barrier dysfunction is observed in atherosclerosis [93] and diabetic retinopathy [94]. Endothelial barrier dysfunction has also been described during ischemia/reperfusion injury after pulmonary thrombarterectomy or transplantation and coronary artery desobstruction. Both interventions are associated with high incidence of edema, either pulmonary [95,96] or myocardial [97] edema. Disruption of the endothelial barrier upon ischemia/reperfusion is thought to result from a localized inflammatory reaction initiated by (re-) exposure of ischemic tissue to fresh plasma constituents and high levels of oxygen (radicals).

Although different with respect to pathophysiology and epidemiology, these clinical descriptions uniformly demonstrate that endothelial barrier dysfunction is associated with high morbidity and mortality rates. The lack in effective treatment aimed at reduction or reversal of endothelial barrier dysfunction, may well sustain these rates.

Treatment of sepsis and ARDS

The therapeutic regimens available for sepsis and ARDS at this moment should be described as *'best supportive care'*. For sepsis, the largest gain in treatment has been achieved by the implementation of *'early goal-directed therapy'* [98]. This regimen aims at fast fluid resuscitation, early obtaining of microbial cultures and swift initiation of antibiotic therapy [74], all preferably within one to six hours after the diagnosis sepsis has been established. Mentioned interventions are designated as *'supportive'* since they either try to prevent development of systemic inflammation from infection (antibiotics) or to control collateral damage (early resuscitation). The same holds true for the treatment modalities available for ARDS. Thus far, medical modesty appeared most effective in the approach to the patient with ARDS, as the largest reduction in ARDS mortality was achieved by implementation of low-tidal volume ventilation [99] and conservative fluid management [100].

When it comes to pharmacological interventions in pathophysiological processes, sepsis has often been called the *'graveyard for pharmaceutical industry'* [101], and the same may well apply to ARDS. The scope of pharmacological intervention has been the dysregulated immune response and the subsequent cytokine storm, as supposed key characteristics of sepsis [77]. Yet, suppression of the immune system, either by blocking cytokines or by corticosteroids, has largely failed to show any effect in clinical studies [76]. A very recent example is eritoran, an antagonist that blocks binding of LPS to its cell surface receptor TLR4 [102]. Despite promising preclinical and initial clinical studies, eritoran failed to improve sepsis mortality at 28 days and 1 year in patients with severe sepsis, [103]. It remains questionable therefore, whether the cytokine storm as observed in preclinical studies plays a predominant role in sepsis pathophysiology. Indeed,

recent studies have even suggested that failure of the immune response (or immunoparalysis) underlies sepsis pathology [104]. The focus on immunomodulation in the development of sepsis pharmacologicals, together with the incomplete understanding of the role of the immune system in sepsis, may have boosted the graveyard.

The endothelium as target for sepsis and ARDS treatment

Combining the failure of immunomodulation with the clinical observation of profound microvascular leak in sepsis and ARDS, investigators have criticized the focus on the immune system and proposed to rather focus on agents that improve endothelial barrier function or methods that target endothelial regeneration and repair [2,78]. Drugs that improve barrier function or reverse barrier dysfunction will not only serve sepsis treatment, but also ARDS and other syndromes characterized by endothelial barrier dysfunction. Fundamental study of endothelial barrier regulation has put forward a number of candidates that have consistently and repeatedly been shown to improve barrier function *in vitro* and *in vivo* (nicely reviewed in: [78]). Key candidates include angiopoietin-1 [106] and S₁P [107], endogenous proteins that enhance barrier function. For inhibition of endothelial barrier function, blockage of mediators like RhoA/ROCK [53] and angiopoietin-2 [108] has proven effective, although clinical evidence is still lacking. Lately, activated protein C (Xigris™), an endogenous protein with various protective effects on the endothelial barrier (amongst others enhanced S₁P production and inhibition of endothelial apoptosis) was shown to have beneficial effects on sepsis in both pre-clinical [109,110] and initial clinical studies [111,112]. Yet, this effect could not be reproduced in additional clinical trials [113,114], leading to the withdrawal of Xigris from the market [80]. Besides development of drugs on the basis of pathophysiological pathways, some existing drugs have promising off-target effects on endothelial barrier function. Statins turned out to be effective in attenuation of endothelial barrier dysfunction – amongst others via inhibition of the RhoA/ROCK pathway [115,116] – and have proven to be safe in the ICU setting [117]. The use of off-target effects from existing drugs (drug-repositioning) offers great advantages over the development of new pharmacologicals [118]. The redundancy of phase I trials and the ample clinical experience with existing drugs, may save both time and money.

Imatinib, tyrosine kinases and endothelial barrier function

More recently, attention was drawn to imatinib (Gleevec®), a small molecule inhibitor designed to treat chronic myeloid leukaemia [119]. In a case-report, initiation of imatinib treatment in a patient with severe pulmonary edema was associated with very fast improvement of oxygenation (<24hour) and resolution of pulmonary edema [120]. A similar effect was reported for a patient

with bleomycin-induced acute lung injury, who recovered after initiation of imatinib treatment [121]. The mechanism by which imatinib mesylate reduced vascular leak remained elusive. Imatinib was designed to inhibit BcrAbl, a constitutively active form of the tyrosine kinase c-Abl, due to its fusion with part of the Bcr gene. Besides c-Abl and BcrAbl, imatinib is known to inhibit Abl-related gene (a kinase with close gene homology to c-Abl), PDGFR- α and - β , c-KIT and DDR-1 [122]. The tyrosine kinase c-Abl was reported to be required for proper endothelial barrier function [123]. Little is known about the role of the other imatinib-sensitive kinases in endothelial barrier regulation.

Diagnosis and outcome prediction in ARDS

Since the first description of ARDS by Ashbaugh in 1967 [124] the definition and diagnosis of ARDS has remained a matter of ongoing debate. The various clinical criteria proposed – within 25 years at least three different criteria (Lung Injury Score, AECC criteria, Berlin criteria) have been proposed – and the critics on these criteria [125,126] all illustrate the difficulty to grasp ARDS. Adequate diagnosis requires on the one hand a satisfying definition that does justice to pathophysiology, and on the other hand clinical criteria that correlate well with the definition. ARDS is a syndrome – in a literal sense ‘a collection of symptoms’. Part of these symptoms come back in the clinical criteria. Endothelial barrier dysfunction and vascular leak are not found in these criteria. The key golden standard for ARDS diagnosis is, however, the presence of hyaline sheets on autopsy findings [126,127], which reflects vascular leakage of fibrinogen to the alveoli. It has been suggested that addition of parameters of vascular leak to the existing clinical criteria may improve accuracy of the ARDS diagnosis [128].

HYPOTHESIS

Altogether, these facts stress the essential role of endothelial barrier function for normal fluid homeostasis. Disruption of the endothelial barrier impairs circulation and organ function, and contributes to morbidity and mortality in the critically ill. Considering the relevance of endothelial barrier regulation in disorders like sepsis and ARDS we hypothesized that: 1) mechanisms of endothelial barrier disruption yield targets for treatment of sepsis and ARDS, and 2) clinical parameters of endothelial barrier disruption improve recognition of sepsis and ARDS. Concerning the clinical recognition of endothelial barrier disruption, this thesis predominantly focused on ARDS.

AIMS OF THE STUDY

1. In general, to evaluate whether mechanisms of endothelial barrier dysfunction may yield targets for treatment of vascular leak and edema formation.
2. Specifically, to address the effect of imatinib on endothelial barrier function, and the role of imatinib-sensitive kinases in endothelial barrier regulation.
3. To reconsider the contribution of cell-matrix interaction to endothelial barrier regulation.
4. Specifically, to study the role of Abl-related gene in endothelial cell-matrix interaction and endothelial barrier regulation.
5. To find out whether clinical parameters of endothelial barrier dysfunction improve the recognition of ARDS.

OUTLINE OF THE THESIS

Chapter 2 provides a thorough review of the literature on the effect of cell-matrix interaction on endothelial barrier function. Specifically we propose a safety mechanism in which fortification of cell-matrix adhesion compensates the loss of cell-cell interaction.

Tyrosine kinases are known to mediate disruption of the endothelial barrier. In two recent case-reports treatment with the tyrosine kinase inhibitor imatinib was followed by fast resolution of pulmonary edema. The underlying mechanism remained unknown, however. In **Chapter 3** we evaluated the effect of imatinib on endothelial barrier function, and explored the possible tyrosine kinases involved in this effect.

The tyrosine kinase Abl-related gene (ARG) has not been described in endothelial biology before. In **Chapter 4** we analysed the role of ARG in endothelial barrier regulation.

The role of the serine/threonine kinase AMPK in endothelial barrier regulation remains controversial. Part of the discussion may be explained by the different role of AMP in barrier disruption and restoration. In **Chapter 5** we evaluated the role of AMPK in short-term barrier disruption.

During vascular leak, the permeability of the endothelial barrier for proteins increases, leading to loss of plasma proteins from the circulation to the surrounding interstitium. It is unclear whether the degree of protein loss is associated with disease severity. In **Chapter 6** we determined the

predictive value of the plasma proteins albumin and transferrin for severity of vascular leak and severity of lung injury.

A lot of effort has been put in the development of biomarkers for ARDS. Biomarkers may improve ARDS diagnostics if readily available and highly predictive for ARDS. Patient plasma is readily available as blood withdrawal is part of standard ICU patient care. The last two decades have yielded many biomarker studies, in particular plasma biomarkers. The relative performance of these biomarkers remains unclear however. Performing a meta-analysis of available literature (**Chapter 7**), we compared the performance of several plasma biomarkers in the prediction of ARDS diagnosis and mortality. The ranking of biomarkers provided in this chapter may guide future research after ARDS diagnosis.

Depending on severity, endothelial barrier disruption and vascular leak are associated with significant fluid loss and hypotension. Following early, goal-directed therapy, fluid loading may restore circulation in cases of shock. However, fluid loading in patients with disturbed barrier function may also have adverse events, leading to congestion and enhanced pulmonary edema. **Chapter 8** searches for predictors of pulmonary edema formation during fluid loading in the critically ill.

Chapter 9 considers the future implications of this thesis with help of two case-reports. The patient data described here illustrate how experimental data may have direct implications and applications in the clinic. Finally, in **Chapter 10** the results of this thesis are discussed. In particular we elaborate on how this thesis may contribute to the understanding of endothelial barrier regulation in the context, and its consequences for treatment and recognition of diseases characterized by endothelial barrier dysfunction.

REFERENCES

1. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med.* 2001;29:1303-10.
2. Lee WL, Slutsky AS. Sepsis and endothelial permeability. *N Engl J Med.* 2010;363:689-91.
3. Shapiro NI, Aird WC. Sepsis and the broken endothelium. *Crit Care.* 2011;15:135.
4. Matthay MA, Ware LB, Zimmerman GA. The acute respiratory distress syndrome. *J Clin Invest.* 2012;122:2731-40.
5. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms.
6. Hwa C, Aird WC. The history of the capillary wall: doctors, discoveries, and debates. *Am J Physiol Heart Circ Physiol.* 2007;293:H2667-79.
7. Wolinsky H. A proposal linking clearance of circulating lipoproteins to tissue metabolic activity as a basis for understanding atherogenesis. *Circ Res.* 1980;47:301-11.
8. Pries AR, Secomb TW, Gaehtgens P. The endothelial surface layer. *Pflugers Arch.* 2000;440:653-66.
9. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood.* 1998;91:3527-61.
10. Ingber DE. Mechanical signaling and the cellular response to extracellular matrix in angiogenesis and cardiovascular physiology. *Circ Res.* 2002;91:877-87.
11. Dejana E, Giampietro C. Vascular endothelial-cadherin and vascular stability. *Curr Opin Hematol.* 2012;19:218-23.
12. Lampugnani MG, Orsenigo F, Rudini N, Maddaluno L, Boulday G, Chapon F, Dejana E. CCM1 regulates vascular-lumen organization by inducing endothelial polarity. *J Cell Sci.* 2010;123:1073-80.
13. Tudor RM, Cool CD, Yeager M, Taraseviciene-Stewart L, Bull TM, Voelkel NF. The pathobiology of pulmonary hypertension. *Endothelium. Clin Chest Med.* 2001;22:405-18.
14. Reinhart-King CA, Dembo M, Hammer DA. The dynamics and mechanics of endothelial cell spreading. *Biophys J.* 2005;89:676-89.
15. Krishnan R, Klumpers DD, Park CY, Rajendran K, Treppe X, van Bezu J, van Hinsbergh VW, Carman CV, Brain JD, Fredberg JJ, Butler JP, van Nieuw Amerongen GP. Substrate stiffening promotes endothelial monolayer disruption through enhanced physical forces. *Am J Physiol Cell Physiol.* 2011;300:C146-54.
16. Langille BL, Adamson SL. Relationship between blood flow direction and endothelial cell orientation at arterial branch sites in rabbits and mice. *Circ Res.* 1981;48:481-8.
17. Chien S. Effects of disturbed flow on endothelial cells. *Ann Biomed Eng.* 2008;36:554-62.
18. Vestweber D. Relevance of endothelial junctions in leukocyte extravasation and vascular permeability. *Ann N Y Acad Sci.* 2012;1257:184-92.
19. Doggett TM, Breslin JW. Study of the actin cytoskeleton in live endothelial cells expressing GFP-actin. *J Vis Exp.* 2011;(57). pii: 3187.
20. Millán J, Cain RJ, Reglero-Real N, Bigarella C, Marcos-Ramiro B, Fernández-Martín L, Correas I, Ridley AJ. Adherens junctions connect stress fibres between adjacent endothelial cells. *BMC Biol.* 2010;8:11.
21. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. *Physiol Rev.* 2006;86:279-367.
22. de Wit C, Hoepfl B, Wölfle SE. Endothelial mediators and communication through vascular gap junctions. *Biol Chem.* 2006;387:3-9.
23. Steed E, Balda MS, Matter K. Dynamics and functions of tight junctions. *Trends Cell Biol.* 2010;20:142-9.

24. Vestweber D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler Thromb Vasc Biol.* 2008;28:223-32.
25. Schulte D, Küppers V, Dartsch N, Broermann A, Li H, Zarbock A, Kamenyeva O, Kiefer F, Khandoga A, Massberg S, Vestweber D. Stabilizing the VE-cadherin-catenin complex blocks leukocyte extravasation and vascular permeability. *EMBO J.* 2011;30:4157-70.
26. Dejana E, Orsenigo F, Lampugnani MG. The role of adherens junctions and VE-cadherin in the control of vascular permeability. *J Cell Sci.* 2008;121:2115-22.
27. Sarai K, Shikata K, Shikata Y, Omori K, Watanabe N, Sasaki M, Nishishita S, Wada J, Goda N, Kataoka N, Makino H. Endothelial barrier protection by FTY720 under hyperglycemic condition: involvement of focal adhesion kinase, small GTPases, and adherens junction proteins. *Am J Physiol Cell Physiol.* 2009;297:C945-54.
28. Huvencers S, Oldenburg J, Spanjaard E, van der Krogt G, Grigoriev I, Akhmanova A, Rehmann H, de Rooij J. Vinculin associates with endothelial VE-cadherin junctions to control force-dependent remodeling. *J Cell Biol.* 2012;196:641-52.
29. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell.* 2002;110:673-87.
30. Giltay JC, van Mourik JA. Structure and function of endothelial cell integrins. *Haemostasis.* 1988;18:376-89.
31. Romer LH, Birukov KG, Garcia JG. Focal adhesions: paradigm for a signaling nexus. *Circ Res.* 2006;98:606-16.
32. Lampugnani, M.G., M. Resnati, E. Dejana, and P.C. Marchisio. The role of integrins in the maintenance of endothelial monolayer integrity. *J Cell Biol.* 1991; 112: 479-90.
33. Sakamoto Y, Ogita H, Hirota T, Kawakatsu T, Fukuyama T, Yasumi M, Kanzaki N, Ozaki M, Takai Y. Interaction of integrin alpha(v)beta3 with nectin. Implication in cross-talk between cell-matrix and cell-cell junctions. *J Biol Chem.* 2006;281:19631-44.
34. Ozaki M, Ogita H, Takai Y. Involvement of integrin-induced activation of protein kinase C in the formation of adherens junctions. *Genes Cells.* 2007;12:651-62.
35. Goeckeler ZM, Wysolmerski RB. Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. *J Cell Biol.* 1995;130:613-27.
36. Shen Q, Rigor RR, Pivetti CD, Wu MH, Yuan SY. Myosin light chain kinase in microvascular endothelial barrier function. *Cardiovasc Res.* 2010;87:272-80.
37. Majno G, Shea SM, Leventhal M. Endothelial contraction induced by histamine-type mediators: an electron microscopic study. *J Cell Biol.* 1969;42:647-72.
38. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci.* 1998;111:1853-65.
39. Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresch DA. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell.* 1999;4:915-24.
40. Vouret-Craviari V, Bourcier C, Boulter E, van Obberghen-Schilling E. Distinct signals via Rho GTPases and Src drive shape changes by thrombin and sphingosine-1-phosphate in endothelial cells. *J Cell Sci.* 2002;115:2475-84.
41. Bradley WD, Koleske AJ. Regulation of cell migration and morphogenesis by Abl-family kinases: emerging mechanisms and physiological contexts. *J Cell Sci.* 2009;122:3441-54.
42. Peacock JG, Couch BA, Koleske AJ. The Abl and Arg non-receptor tyrosine kinases regulate different zones of stress fiber, focal adhesion, and contractile network localization in spreading fibroblasts. *Cytoskeleton (Hoboken).* 2010;67:666-75.
43. Stahlmann N, Woods A, Carling D, Heller R. Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca²⁺/calmodulin-dependent protein kinase kinase beta. *Mol Cell Biol.* 2006; 26:5933-45.

44. Fisslthaler B, Loot AE, Mohamed A, Busse R, Fleming I. Inhibition of endothelial nitric oxide synthase activity by proline-rich tyrosine kinase 2 in response to fluid shear stress and insulin. *Circ Res*. 2008;102:1520-8.
45. Bone RC. The Pathogenesis of Sepsis. *Ann Intern Med*. 1991;115:457-469.
46. Taylor FB Jr, Emerson TE Jr, Jordan R, Chang AK, Blick KE. Antithrombin-III prevents the lethal effects of *Escherichia coli* infusion in baboons. *Circ Shock*. 1988;26:227-35.
47. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000;407:258-64.
48. van der Heijden M, van Nieuw Amerongen GP, Koolwijk P, van Hinsbergh VW, Groeneveld AB. Angiopoietin-2, permeability oedema, occurrence and severity of ALI/ARDS in septic and non-septic critically ill patients. *Thorax*. 2008;63:903-9.
49. Agrawal A, Matthay MA, Kangelaris KN, Stein J, Chu JC, Imp BM, Cortez A, Abbott J, Liu KD, Calfee CS. Plasma angiopoietin-2 predicts the onset of acute lung injury in critically ill patients. *Am J Respir Crit Care Med*. 2013;187:736-42.
50. Gallagher DC, Parikh SM, Balonov K, Miller A, Gautam S, Talmor D, Sukhatme VP. Circulating angiopoietin 2 correlates with mortality in a surgical population with acute lung injury/adult respiratory distress syndrome. *Shock*. 2008;29:656-61.
51. Chen CY, Yang KY, Chen MY, Chen HY, Lin MT, Lee YC, Perng RP, Hsieh SL, Yang PC, Chou TY. Decoy receptor 3 levels in peripheral blood predict outcomes of acute respiratory distress syndrome. *Am J Respir Crit Care Med*. 2009;180:751-60.
52. Tirupathi C, Malik AB, Del Vecchio PJ, Keese CR, Giaever I. Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. *Proc Natl Acad Sci U S A*. 1992;89:7919-23.
53. van Nieuw Amerongen GP, Draijer R, Vermeer MA, van Hinsbergh VW. Transient and prolonged increase in endothelial permeability induced by histamine and thrombin: role of protein kinases, calcium, and RhoA. *Circ Res*. 1998;83:1115-23.
54. Weis SM, Cheresh DA. Pathophysiological consequences of VEGF-induced vascular permeability. *Nature*. 2005;437:497-504.
55. Starling EH. On the Absorption of Fluids from the Connective Tissue Spaces. *J Physiol*. 1896;19:312-26.
56. Rose B, Post TW. *Clinical Physiology of Acid-Base and Electrolyte Disorders*. McGraw-Hill Professional. 2001.
57. Staub NC. Pulmonary edema: physiologic approaches to management. *Chest*. 1978;74:559-64.
58. Levick JR, Michel CC. Microvascular fluid exchange and the revised Starling principle. *Cardiovasc Res*. 2010;87:198-210.
59. Levick JR, Michel CC. The effects of position and skin temperature on the capillary pressures in the fingers and toes. *J Physiol*. 1978; 274:97-109.
60. Adamson RH, Lenz JF, Zhang X, Adamson GN, Weinbaum S, Curry FE. Oncotic pressures opposing filtration across non-fenestrated rat microvessels. *J Physiol*. 2004; 557:889-907.
61. Schmidt EP, Yang Y, Janssen WJ, Gandjeva A, Perez MJ, Barthel L, Zemans RL, Bowman JC, Koyanagi DE, Yunt ZX, Smith LP, Cheng SS, Overdier KH, Thompson KR, Geraci MW, Douglas IS, Pearse DB, Tudor RM. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. *Nat Med*. 2012;18.
62. Woodcock TE, Woodcock TM. Revised Starling equation and the glycocalyx model of transvascular fluid exchange: an improved paradigm for prescribing intravenous fluid therapy. *Br J Anaesth*. 2012;108:384-94.
63. Toska K. Letter by Toska regarding article, "Biological basis of therapeutic lymphangiogenesis". *Circulation*. 2011;124:e895.
64. Staub NC. Pulmonary edema. *Physiol Rev*. 1974;54:678-811.
65. Wheeler AP, Bernard GR. Treating Patients with Severe Sepsis. *N Engl J Med* 1999; 340:207-214.

66. Yano K, Liaw PC, Mullington JM, Shih SC, Okada H, Bodyak N, Kang PM, Toltl L, Belikoff B, Buras J, Simms BT, Mizgerd JP, Carmeliet P, Karumanchi SA, Aird WC. Vascular endothelial growth factor is an important determinant of sepsis morbidity and mortality. *J Exp Med*. 2006;203:1447-58.
67. London NR, Zhu W, Bozza FA, Smith MC, Greif DM, Sorensen LK, Chen L, Kaminoh Y, Chan AC, Passi SF, Day CW, Barnard DL, Zimmerman GA, Krasnow MA, Li DY. Targeting Robo4-dependent Slit signaling to survive the cytokine storm in sepsis and influenza. *Sci Transl Med*. 2010;2:23ra19.
68. Teijaro JR, Walsh KB, Cahalan S, Fremgen DM, Roberts E, Scott F, Martinborough E, Peach R, Oldstone MB, Rosen H. Endothelial cells are central orchestrators of cytokine amplification during influenza virus infection. *Cell*. 2011;146:980-91.
69. Timmerman I, Hoogenboezem M, Bennett AM, Geerts D, Hordijk PL, van Buul JD. The tyrosine phosphatase SHP2 regulates recovery of endothelial adherens junctions through control of β -catenin phosphorylation. *Mol Biol Cell*. 2012;23:4212-25.
70. Profirovic J, Han J, Andreeva AV, Neamu RF, Pavlovic S, Vogel SM, Walter U, Voyno-Yasenetskaya TA. Vasodilator-stimulated phosphoprotein deficiency potentiates PAR-1-induced increase in endothelial permeability in mouse lungs. *J Cell Physiol*. 2011;226:1255-64.
71. Groeneveld AB, Raijmakers PG. The 67 Gallium-transferrin pulmonary leak index in patients at risk for the acute respiratory distress syndrome. *Crit Care Med*. 1998;26:685-91.
72. Chung FT, Lin HC, Kuo CH, Yu CT, Chou CL, Lee KY, Kuo HP, Lin SM. Extravascular lung water correlates multiorgan dysfunction syndrome and mortality in sepsis. *PLoS One*. 2010;5:e15265.
73. Lever A, Mackenzie I. Sepsis: definition, epidemiology, and diagnosis. *BMJ*. 2007;335:879-83.
74. Dellinger RP, Levy MM, Carlet JM, Bion J, Parker MM, Jaeschke R, Reinhart K et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Crit Care Med*. 2008;36:296-327.
75. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G; SCCM/ESICM/ACCP/ATS/SIS. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med*. 2003;31:1250-6.
76. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med*. 2003;348:138-50.
77. Cohen J. The immunopathogenesis of sepsis. *Nature*. 2004;420:885-91.
78. Goldenberg NM, Steinberg BE, Slutsky AS, Lee WL. Broken barriers: a new take on sepsis pathogenesis. *Sci Transl Med*. 2011;3:88ps25.
79. van Gestel A, Bakker J, Veraart CP, van Hout BA. Prevalence and incidence of severe sepsis in Dutch intensive care units. *Crit Care*. 2004;8:R153-62.
80. Lancet Infectious Diseases Editor. For sepsis, the drugs don't work. *Lancet Infectious Dis*. 2012;12:89.
81. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, LeGall JR, Morris A, Spragg R. Report of the American-European consensus conference on ARDS: definitions, mechanisms, relevant outcomes and clinical trial coordination. The Consensus Committee. *Intensive Care Med*. 1994;20:225-32.
82. ARDS Definition Task Force, Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS. Acute respiratory distress syndrome: the Berlin Definition. *JAMA*. 2012;307:2526-33.
83. Raijmakers PG, Groeneveld AB, Teule GJ, Thijs LG. Diagnostic value of the gallium-67 pulmonary leak index in pulmonary edema. *J Nucl Med*. 1996;37:1316-22.
84. Brown LM, Liu KD, Matthay MA. Measurement of extravascular lung water using the single indicator method in patients: research and potential clinical value. *Am J Physiol Lung Cell Mol Physiol*. 2009;297:L547-58.
85. Li G, Malinchoc M, Cartin-Ceba R, Venkata CV, Kor DJ, Peters SG, Hubmayr RD, Gajic O. Eight-year trend of acute respiratory distress syndrome: a population-based study in Olmsted County, Minnesota. *Am J Respir Crit Care Med*. 2011;183:59-66.

86. Luhr OR, Antonsen K, Karlsson M, Aardal S, Thorsteinsson A, Frostell CG, Bonde J. Incidence and mortality after acute respiratory failure and acute respiratory distress syndrome in Sweden, Denmark, and Iceland. The ARF Study Group. *Am J Respir Crit Care Med.* 1999;159:1849-61.
87. Wind J, Versteegt J, Twisk J, van der Werf TS, Bindels AJ, Spijkstra JJ, Girbes AR, Groeneveld AB. Epidemiology of acute lung injury and acute respiratory distress syndrome in The Netherlands: a survey. *Respir Med.* 2007;101:2091-8.
88. Spragg RG, Bernard GR, Checkley W, Curtis JR, Gajic O, Guyatt G, Hall J, Israel E, Jain M, Needham DM, Randolph AG, Rubenfeld GD, Schoenfeld D, Thompson BT, Ware LB, Young D, Harabin AL. Beyond mortality: future clinical research in acute lung injury. *Am J Respir Crit Care Med.* 2010;181:1121-7.
89. Druey KM, Greipp PR. Narrative review: the systemic capillary leak syndrome. *Ann Intern Med.* 2010;153:90-8.
90. Xie Z, Ghosh CC, Patel R, Iwaki S, Gaskins D, Nelson C, Jones N, Greipp PR, Parikh SM, Druey KM. Vascular endothelial hyperpermeability induces the clinical symptoms of Clarkson disease (the systemic capillary leak syndrome). *Blood.* 2012;119:4321-32.
91. Korhonen H, Fisslthaler B, Moers A, Wirth A, Habermehl D, Wieland T, Schütz G, Wettchuck N, Fleming I, Offermanns S. Anaphylactic shock depends on endothelial Gq/G11. *J Exp Med.* 2009;206:411-20.
92. Czúcz J, Schaffer G, Csuka D, Walentin S, Kunde J, Prohászka Z, Farkas H, Cervenak L. Endothelial cell function in patients with hereditary angioedema: elevated soluble E-selectin level during inter-attack periods. *J Clin Immunol.* 2012; 32:61-9.
93. Sun C, Wu MH, Lee ES, Yuan SY. A disintegrin and metalloproteinase 15 contributes to atherosclerosis by mediating endothelial barrier dysfunction via Src family kinase activity. *Arterioscler Thromb Vasc Biol.* 2012; 32:2444-51.
94. Lang GE. Diabetic macular edema. *Ophthalmologica.* 2012; 227 Suppl 1:21-9.
95. Levinson RM, Shure D, Moser KM. Reperfusion pulmonary edema after pulmonary artery thromboendarterectomy. *Am Rev Respir Dis.* 1986;134:1241-5.
96. Miller WT Jr, Osiason AW, Langlotz CP, Palevsky HI. Reperfusion edema after thromboendarterectomy: radiographic patterns of disease. *J Thorac Imaging.* 1998;13:178-83.
97. Garcia-Dorado D, Andres-Villarreal M, Ruiz-Meana M, Inserte J, Barba I. Myocardial edema: a translational view. *J Mol Cell Cardiol.* 2012;52:931-9.
98. Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, Peterson E, Tomlanovich M; Early Goal-Directed Therapy Collaborative Group. Early goal-directed therapy in the treatment of severe sepsis and septic shock. *N Engl J Med.* 2001;345:1368-77.
99. The Acute Respiratory Distress Syndrome Network. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. *N Engl J Med.* 2000;342:1301-8.
100. National Heart, Lung, and Blood Institute Acute Respiratory Distress Syndrome (ARDS) Clinical Trials Network, Wiedemann HP, Wheeler AP, Bernard GR, Thompson BT, Hayden D, deBoisblanc B, Connors AF Jr, Hite RD, Harabin AL. Comparison of two fluid-management strategies in acute lung injury. *N Engl J Med.* 2006;354:2564-75.
101. Riedemann NC, Guo RF, Ward PA. Novel strategies for the treatment of sepsis. *Nat Med.* 2003;9:517-24.
102. Barochia A, Solomon S, Cui X, Natanson C, Eichacker PQ. Eritoran tetrasodium (E5564) treatment for sepsis: review of preclinical and clinical studies. *Expert Opin Drug Metab Toxicol.* 2011;7:479-94.
103. Opal SM, Laterre PF, Francois B, LaRosa SP, Angus DC, Mira JP, Wittebole X, Dugernier T, Perrotin D et al; ACCESS Study Group. Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial. *JAMA.* 2013;309:1154-62.
104. Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, Bricker TL, Jarman SD 2nd, Kreisel D, Krupnick AS, Srivastava A, Swanson PE, Green JM, Hotchkiss RS. Immunosuppression in patients who die of sepsis and multiple organ failure. *JAMA.* 2011;306:2594-605.

105. Leentjens J, Kox M, Koch RM, Preijers F, Joosten LA, van der Hoeven JG, Netea MG, Pickkers P. Reversal of immunoparalysis in humans in vivo: a double-blind, placebo-controlled, randomized pilot study. *Am J Respir Crit Care Med*. 2012;186:838-45.
106. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med*. 2000;6:460-3.
107. Camerer E, Regard JB, Cornelissen I, Srinivasan Y, Duong DN, Palmer D, Pham TH, Wong JS, Pappu R, Coughlin SR. Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. *J Clin Invest*. 2009;119:1871-9.
108. Parikh SM, Mammoto T, Schultz A, Yuan HT, Christiani D, Karumanchi SA, Sukhatme VP. Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. *PLoS Med*. 2006;3:e46.
109. Taylor FB Jr, Chang A, Esmon CT, D'Angelo A, Vigano-D'Angelo S, Blick KE. Protein C prevents the coagulopathic and lethal effects of *Escherichia coli* infusion in the baboon. *J Clin Invest*. 1987;79:918-25.
110. Roback MG, Stack AM, Thompson C, Brugnara C, Schwarz HP, Saladino RA. Activated protein C concentrate for the treatment of meningococcal endotoxin shock in rabbits. *Shock*. 1998;9:138-42.
111. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al; Recombinant human protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study group. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med*. 2001;344:699-709.
112. Vincent JL, Bernard GR, Beale R, Doig C, Putensen C, Dhainaut JF, Artigas A, Fumagalli R, Macias W, Wright T, Wong K, Sundin DP, Turlo MA, Janes J. Drotrecogin alfa (activated) treatment in severe sepsis from the global open-label trial ENHANCE: further evidence for survival and safety and implications for early treatment. *Crit Care Med*. 2005;33:2266-77.
113. Nadel S, Goldstein B, Williams MD, Dalton H, Peters M, Macias WL, Abd-Allah SA, Levy H, Angle R, Wang D, Sundin DP, Giroir B; REsearching severe Sepsis and Organ dysfunction in children: a gLobal perspective (RESOLVE) study group. Drotrecogin alfa (activated) in children with severe sepsis: a multicentre phase III randomised controlled trial. *Lancet*. 2007;369:836-43.
114. Ranieri VM, Thompson BT, Barie PS, Dhainaut JF, Douglas IS, Finfer S, Gårdlund B, Marshall JC, Rhodes A, Artigas A, Payen D, Tenhunen J, Al-Khalidi HR, Thompson V, Janes J, Macias WL, Vangerow B, Williams MD; PROWESS-SHOCK Study Group. Drotrecogin alfa (activated) in adults with septic shock. *N Engl J Med*. 2012;366:2055-64.
115. van Nieuw Amerongen GP, Vermeer MA, Nègre-Aminou P, Lankelma J, Emeis JJ, van Hinsbergh VW. Simvastatin improves disturbed endothelial barrier function. *Circulation*. 2000;102:2803-9.
116. Singla S, Jacobson JR. Statins as a novel therapeutic strategy in acute lung injury. *Pulm Circ*. 2012; 2:397-406.
117. van de Visse EP, van der Heijden M, Verheij J, van Nieuw Amerongen GP, van Hinsbergh VW, Girbes AR, Groeneveld AB. Effect of prior statin therapy on capillary permeability in the lungs after cardiac or vascular surgery. *Eur Respir J*. 2006; 27:1026-32.
118. Sardana D, Zhu C, Zhang M, Gudivada RC, Yang L, Jegga AG. Drug repositioning for orphan diseases. *Brief Bioinform*. 2011;12:346-56.
119. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031-7.
120. Overbeek MJ, van Nieuw Amerongen GP, Boonstra A, Smit EF, Vonk-Noordegraaf A. Possible role of imatinib in clinical pulmonary veno-occlusive disease. *Eur Respir J*. 2008;32:232-5.
121. Carnevale-Schianca F, Gallo S, Rota-Scalabrini D, Sangiolo D, Fizzotti M, Caravelli D, Capaldi A, Anselmetti G, Palesandro E, D'Ambrosio L, Coia V, Obert R, Aglietta M, Grignani G. Complete resolution of life-threatening bleomycin-induced pneumonitis after treatment with imatinib mesylate in a patient with Hodgkin's lymphoma: hope for severe chemotherapy-induced toxicity? *J Clin Oncol*. 2011;29:e691-3.

122. Waller CF. Imatinib mesylate. *Recent Results Cancer Res.* 2010; 184:3–20.
123. Dudek SM, Chiang ET, Camp SM, Guo Y, Zhao J, Brown ME, Singleton PA, Wang L, Desai A, Arce FT, Lal R, Van Eyk JE, Imam SZ, Garcia JG. Abl tyrosine kinase phosphorylates nonmuscle myosin light chain kinase to regulate endothelial barrier function. *Mol Biol Cell.* 2010;21:4042-4056.
124. Ashbaugh DG, Bigelow DB, Petty TL, et al. Acute respiratory distress in adults. *Lancet.* 1967;2:319-23.
125. Estenssoro E, Dubin A, Laffaire E, et al. Impact of positive end-expiratory pressure on the definition of acute respiratory distress syndrome. *Intensive Care Med.* 2003;29:1936-42.
126. Esteban A, Fernández-Segoviano P, Frutos-Vivar F, Aramburu JA, Nájera L, Ferguson ND, Alía I, Gordo F, Ríos F. Comparison of clinical criteria for the acute respiratory distress syndrome with autopsy findings. *Ann Intern Med.* 2004;141:440-5.
127. Tomaszewski JF Jr. Pulmonary pathology of acute respiratory distress syndrome. *Clin Chest Med.* 2000;21:435-66.
128. Perel A. Extravascular lung water and the pulmonary vascular permeability index may improve the definition of ARDS. *Crit Care.* 2013;17:108.

