Chapter 10

General Discussion
Disruption of the endothelial barrier and the consequent vascular leak play a dominant role in the pathophysiology of sepsis and ARDS. With focus on endothelial barrier regulation as a dynamic process, we have sought to identify targets for treatment of vascular leak and ways to recognize permeability-driven edema. The first part of the Discussion involves studies on mechanisms of endothelial barrier regulation, with specific emphasis on endothelial cell-matrix interaction. In the second part, we address the translation of identified targets to functional therapies for diseases characterized by endothelial barrier dysfunction and vascular leak, while the third part addresses the development of strategies to recognize vascular leak.

PART I – NOVEL MECHANISMS OF ENDOTHELIAL BARRIER REGULATION

1.1 – Contribution of cell-matrix interaction to endothelial barrier dysfunction

An important question addressed in this thesis is how cell-matrix interaction contributes to endothelial barrier regulation. Reviewing the current literature (Chapter 2), we provide evidence for the hypothesis that artificial modification of proteins responsible for cell-matrix interaction (integrins, focal adhesion proteins like FAK and talin) alters the integrity of the endothelial barrier. Knockdown or functional interference with blocking antibodies of for instance α5β1 integrin severely impairs endothelial barrier function. Similar results were found for αvβ3 integrin, although its exact contribution to endothelial barrier function may vary per condition (Chapter 2). These data stress the relevance of cell-matrix adhesion per se for the (maintenance of) integrity of the endothelial monolayer.

It remains elusive, however, whether (during endothelial stimulation with barrier-disruptive agents) modification of cell-matrix interaction as an active process contributes to endothelial barrier disruption. Particular attention should be paid here to integrins, since these proteins form the anatomical core of cell-matrix interaction [1]. Intracellular signaling triggered by binding of vasoactive agents to the cell surface may alter integrin avidity and affinity for the extracellular matrix. Little is known about this ‘inside-out’ integrin signaling as part of the barrier-disruptive events during inflammation. Theoretically, various scenarios are possible and have been suggested in the literature: 1) integrins are not actively involved, and cell-matrix interaction is subjected to active changes in cytoskeleton and cell-cell contacts, 2) integrin activation contributes to barrier disruption, for instance by forming anchoring points for contractile fibers [2,3], 3) integrin activation improves cell-matrix interaction as a protective reflex to maintain cell shape, thereby limiting cell contraction and barrier disruption [4,5], 4) a mixed scenario in which some integrins contribute to barrier disruption by forming anchoring points for contractile fibers and other integrins improve cell matrix interaction and maintain cell shape. Integrin function in this mixed

2 In this case, passive force of cell-matrix interaction may still withstand cell contraction.
scenario may depend on integrin heterodimer subtype or subcellular localization (central versus peripheral). In our studies on imatinib (Chapter 3) we observed that imatinib treatment leads to peripheral redistribution of focal adhesions during thrombin stimulation. Imatinib-pretreated cells showed a large number of focal adhesions close to cell borders. These events were paralleled by attenuation of endothelial barrier disruption in vitro and in vivo. The protective effects of imatinib were predominantly mediated by inhibition of the tyrosine kinase ARG, a kinase known to be involved in spatial distribution of focal adhesions [9]. In Chapter 4 we demonstrate that ARG plays an important role in the expression and distribution of integrins $\alpha_5\beta_1$. Knockdown of ARG enhanced protein expression of $\alpha_5\beta_1$ integrin and resulted in redistribution of $\alpha_5\beta_1$ integrin to the cell borders. This was associated with increased adhesional strength of endothelial cells to the extracellular matrix, suggesting that ARG decreases cell-matrix interaction, in particular at the cell margins. Knockdown of ARG in human endothelial cells or genetic depletion of ARG in mice attenuated endothelial barrier disruption by vaso-active agents. Chapter 3 and Chapter 4 therefore show that ARG regulates the distribution of focal adhesions and integrins both in a resting monolayer and during endothelial barrier dysfunction. These data provide evidence for active regulation of cell-matrix interaction as part of the barrier-disruptive events during inflammation. In short, ARG is activated during stimulation with barrier-disruptive agents and inhibits the formation of peripheral adherional complexes at the cell periphery. Given the protective effects of imatinib and ARG knockdown on endothelial barrier disruption that parallel this peripheral redistribution of cell-matrix adhesions, we wondered whether active regulation of cell-matrix interaction at the cell borders is a key determinant of interendothelial gap formation, endothelial barrier dysfunction and vascular leak.

This hypothesis is supported by the literature and structural analysis of interendothelial gap formation. Ultrastructural analyses of endothelial cells by electron microscopy have shown detachment of endothelial cells upon stimulation with barrier-disruptive agents or in septic conditions. This detachment takes place at the cell borders [10,11], in contrast with the center of the endothelial cells, where cell-matrix interaction seems to be enhanced [12]. The electron microscopy analyses of Majno et al. and Hattori et al. (Figure 1A,C) suggest that dissociation of cell-matrix interaction at the cell periphery contributes to or may even be required for gap formation. Taken together with the vast amount of evidence on the contribution of cell-cell contact to endothelial barrier function [13,14], this leads to a comprehensive mechanistical model of endothelial barrier regulation in which cell-cell contact and cell-matrix adhesion at the cell

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3 A fifth scenario may exist, in which integrins empower cell-cell contacts by a direct effect on junctions (e.g. presence of integrins in cell-cell junctions [6]) or by an indirect effect on junctions (e.g. via GTPases Rac1 and Rap1) [7,8].
borders withstand cell contraction [Figure 1B]. Endothelial barrier dysfunction is characterized by loss of cell-cell contact and loss of peripheral cell-matrix interaction [Chapters 3 and 4] on the one hand, and increased cell contraction on the other [12,15,16]. Thus, release of cell-matrix adhesion at the cell border is a necessary condition for cell contraction and gap formation. Assuming a random cross-section through the gaps shown in Figure 1 (representative for cross-sections in any other given direction), the area of cell detachment from the extracellular matrix is a direct determinant of the size of an interendothelial gap. The work presented in this thesis suggests that ARG is a central regulator of cell-matrix adhesion at the cell border in endothelial cells, and is in line with previous literature on the role of ARG in fibroblasts [6] and epithelial cells [18]. Although certain mechanisms seem to enhance cell-matrix interaction at the cell borders as a protective reflex in the very early stage (1-2 mins after thrombin stimulation) of endothelial barrier disruption [4,19], this reflex is downregulated after activation of ARG (2-5min after thrombin stimulation, Chapter 4). We therefore propose that ARG inhibits reactive fortification of cell-matrix interaction at the cell borders and allows for cell retraction and overt gap formation at a later stage (5-15min). Our finding that ARG is activated upon a wide variety of barrier-disruptive stimuli (VEGF, thrombin, histamine), and that ARG activation seems to be enhanced in the pulmonary vessels (particularly the endothelium) of septic patients suggests that ARG activation is a very general phenomenon during inflammation and endothelial barrier disruption.

Although Chapter 4 does not resolve how ARG regulates integrin distribution, it provides evidence for Rap1 activity as link in the ARG-α5β1 integrin interaction. Pharmacological inhibition and genetic knockdown of ARG enhanced and prolonged Rap1 activity during thrombin stimulation, indicating that during thrombin stimulation ARG contributes to Rap1 inactivation (in particular at 5-10min). Previous studies have shown that ARG activation leads to Rap1 inactivation [18], among others via phosphorylation of the adapter protein CrkL [20]. CrkL phosphorylation at Tyr207 prevents complex formation of CrkL with the Rap1-GEF C3G, leading to Rap1 inactivation [20]. Rap1 was recently shown to contribute to endothelial barrier integrity by enhancing cell spreading and improving cell-cell contacts via the Rap1 downstream effectors Rasip1 and Radil [21]. In addition, Rap1 contributes to endothelial barrier recovery by abrogating RhoA-induced actomyosin contractility after thrombin-induced barrier disruption [22]. As Rap1 is known to enhance cell-matrix interaction in an integrin-dependent manner [23-25], initial Rap1 activity may contribute to protective cell-matrix fortification in the very early stage, which is abrogated when ARG becomes fully active (2-5min), leading to loss of cell-matrix interaction at the cell border at a later stage (see above).

4 In a strict sense, release of cell-matrix adhesion cannot be called necessary, as VE-cadherin blocking antibodies (which are presumed to solely affect VE-cadherin mediated cell-cell contact) have been shown to induce gap formation [17]. Breakdown of cell-cell contact is therefore sufficient for gap formation, precluding release of cell-matrix interaction as necessary condition. As far as we know, however, such external breakdown of cell-cell contacts has not been observed as part of any inflammatory response.
Although several data from the current thesis support this mechanism (see Discussion Chapter 4), additional experiments are required to confirm the direction of causality in this signaling pathway. Alternatively, the barrier disruptive effects of ARG could be explained by its effect on the small GTPase Rac1. In Chapter 3 we show that pharmacological ARG inhibition with imatinib enhanced Rac1 activity, suggesting that ARG in endothelial cells is an inhibitor of Rac1. As Rac1 is known to drive both AJ stabilization [26,27] and cell spreading [28], Rac1 inhibition may well explain the barrier-disruptive effects of ARG. It is also in line with our findings that ARG knockdown results in enhanced cell spreading (Chapter 4). Although Rap1 and Rac1 have both

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5 Chapters 3 and 4 show that: a) thrombin induces phosphorylation of CrkL at Tyr207, which is completely ARG-dependent, b) the sequence and time interval of events in the mechanism proposed match the time frame observed in our experiments, c) ARG knockdown prolongs and enhances Rap1 activity, and d) ARG knock down strongly enhances cell-matrix interaction.
been shown to act downstream ARG in the regulation of epithelial monolayer morphology [18],
the relative contribution of Rap1 or Rac1 to ARG induced endothelial barrier disruption remains
to be determined.

Some (in particular mechanistical) issues remain unresolved here. A first issue that remains
unresolved is how ARG regulates $\alpha_5\beta_1$ protein expression. We demonstrate that ARG knockdown
leads to enhanced expression of $\alpha_5\beta_1$ integrin in endothelial cells. Despite a recently discovered
ARG isoform with nuclear localization [29], ARG does not contain a DNA binding site [30,31].
Therefore it is unlikely that ARG acts as a direct suppressor gene. An alternative explanation is that
ARG contributes to structural/morphological changes that in turn change integrin expression, as
described for Rap1 [24]. Second, it remains elusive at which level ARG interferes with integrin
regulation. Although ARG knockdown enhanced $\alpha_5\beta_1$ integrin expression, long-term incubation
of endothelial cells with imatinib did not (Chapter 4). The enhanced integrin expression per se is
therefore not sufficient to explain the protective effects of ARG knockdown on thrombin-induced
endothelial barrier disruption. The finding that 30-60min of imatinib pretreatment is sufficient
to protect against endothelial barrier disruption (Chapter 3), also indicates that the protective
effects of ARG inhibition are independent of long-term processes like protein expression (hours to
days). Alternatively, ARG may interfere with integrin regulation at the level of spatial distribution
of integrins, integrin expression at the membrane and integrin activation by changes in integrin
conformation. As discussed above, Rap1 and Rac1 may play an important role in these short-term
integrin changes, and deserves further attention in this context. Additional immunofluorescence
imaging and flow cytometry experiments are required to determine the effects of ARG on spatial
distribution and membrane expression of integrins in endothelial cells. The third issue is how the
peripherally redistributed integrins improve endothelial barrier function. Several possibilities exist
here: a) peripheral integrins enhance peripheral cell-matrix interaction, preventing cell retraction,
b) peripheral integrins support cell-cell contacts [5,32], c) peripheral integrins reduce tension in the
cell-cell contacts, or d) peripheral integrins contribute to cell spreading, enhancing recovery of the
barrier. Fourth, the direct effects of ARG on cell-cell junctions and the actin cytoskeleton remain
unclear. We have repeatedly shown that pharmacological inhibition or genetic knockdown of
ARG does not change the expression of AJ proteins like VE-cadherin and $\beta$-catenin (Chapter 4). It
remains to be addressed, however, whether ARG affects phosphorylation of these proteins, since
ARG colocalized and immunoprecipitated with $\beta$-catenin and E-cadherin in epithelial cells [33],
and since tyrosine phosphorylation of VE-cadherin [34,35] and $\beta$-catenin [36,37] may regulate
strength of cell-cell contacts independent of expression. Recent studies suggest that integrin-
mediated adhesions can modulate the composition and tension of cell-cell junctions [38]. We
also did not observe any effect of ARG on the actin cytoskeleton. A similar actin morphology
was observed in control and ARG-inhibited endothelial cells. This finding is surprising, since fibroblast studies have indicated ARG as important regulator of actin [39,40]. ARG contains two actin binding sites [30], and regulates the activity of major actin-regulating proteins like cortactin [41,42] and p190RhoGAP [43,44]. Collectively, these data point toward a novel and significant pathway for endothelial barrier disruption, in which ARG plays a central role. The signaling events and functional consequences of this pathway are summarized in Figure 2A. This model also shows how pharmacological or genetic interference with this pathway may attenuate endothelial barrier disruption and vascular leak (Figure 2B).

**Figure 2** Cartoon of signaling events associated with ARG activity during endothelial barrier dysfunction, as proposed in this thesis. A) Endothelial stimulation with vasoactive agents leads to Src-dependent activation of ARG, which in turn inhibits the small RhoGTPases Rac1 and Rap1, which in turn leads to β₁ integrin orientation at the cell center. B) Pharmacologic and genetic interference with ARG activity reverses or prevents ARG-induced changes, leading to presence of β₁ integrin at the cell perimeter.
1.2 – The role of AMPK in endothelial barrier regulation
AMPK is a serine/threonine kinase, which serves as an energy sensor (AMP/ATP ratio) and regulates energy metabolism of the cell [45]. The exact involvement of AMPK in endothelial barrier disruption remains incompletely understood. In Chapter 5, we show that AMPK is activated during endothelial barrier disruption. Stimulation of endothelial cells with barrier-disruptive agents like thrombin and VEGF leads to AMPK activation in a very early phase (2-5 min after stimulation). Inhibition of AMPK with Compound C dose-dependently attenuated thrombin-induced endothelial barrier disruption. The results of this study therefore suggest that AMPK mediates thrombin-induced endothelial barrier disruption.

The role of AMPK in vascular leak and edema formation has been debated thus far. A number of studies have shown that pharmacological inhibition of AMPK or knockdown of one of either AMPK subunits aggravates ALI phenotype in mice [46,47]. Other studies have shown that AMPK α1 knockdown attenuated ALI development [48]. These paradoxical results may follow from differences in diseases models, a heterogeneous expression pattern of AMPK subunits or differences in inhibitor (Compound C) concentrations. Alternatively, they may point toward a very complex involvement of AMPK in endothelial barrier regulation, which may include a role in both barrier disruption and recovery. Due to the limited understanding of the exact role of AMPK in endothelial barrier regulation the application of AMPK-modulating agents for treatment of vascular leak and edema formation still remains a bridge too far. Key experiments for further elucidation of AMPK in endothelial barrier regulation include testing of Compound C in vivo models of vascular leak. In addition, knockdown of either AMPK α isoforms in cultured endothelial cells and (endothelial-specific) knockout of AMPK in mice, requires analysis for endothelial barrier function. AMPK α1 knockout mice are currently evaluated in the Miles assay in collaboration with the University of Jena (Germany). It is of interest to note that Compound C is a potent inhibitor of Arg [49].

1.3 – Conclusion part I
These studies elucidate novel mechanisms of endothelial barrier disruption, targeting of which attenuates vascular leak and edema formation. First, we provide evidence for the hypothesis that rearrangement of cell-matrix interactions contributes to endothelial barrier disruption, and that active regulation of cell-matrix interaction participates in the cascade of barrier-disruptive events. ARG seems to play a central and essential role in this active regulation of cell-matrix interaction. Second, we add to the existing controversy on the role of AMPK in endothelial barrier regulation by showing that AMPK contributes to barrier disruption. Since kinase activity of both ARG and AMPK seem to be at stake during endothelial barrier disruption, and since both kinases are
adequately targeted by existing pharmacological compounds, these studies may contribute to
target-directed development of therapy.

PART II – TREATMENT OF ENDOTHELIAL BARRIER DYSFUNCTION

Having identified targets in endothelial barrier disruption, the next step towards clinically
applicable treatment regimens involves the development of specific interventions. One particular
pharmacokinetic advantage of treating endothelial derangements like vascular leak, is that a given
drug (when administered intravenously) directly hits the target organ (c.q. the endothelium), and
does not require characteristics for high tissue penetration. For the same reason it also allows for
lower drug dosing to obtain sufficient drug concentration at the target organ. Interventions for
targets like the kinases ARG and AMPK include either pharmacological inhibition (small molecule
inhibitors) or genetic knockdown (interfering RNA). Small molecules usually show fast inhibition
of the target, but their use is complicated by lack of specificity and off-target effects. Genetic
knockdown, in contrast, has high target specificity, but takes considerable time before biological
effect is obtained; knockdown of target protein takes hours to days. The sequence of events in
the development of specific intervention involves: 1) exploitation of existing drugs and chemical
libraries, 2) development of novel inhibitors based on the protein structure of identified targets
(e.g. ARG), and 3) development of genetic interference based on genetic sequence of the target.

2.1 – Development of target-specific inhibitors (small molecules)
At present imatinib is recognized as an effective pharmacological inhibitor of ARG. Taking CrkL
(Tyr207) phosphorylation as read-out for ARG activity, we found that imatinib completely inhibits
Tyr207 CrkL phosphorylation (Chapter 3), confirming the effectivity of imatinib as ARG inhibitor
in endothelial cells. Several characteristics of imatinib argue in favour of its development as
therapy for vascular leak: 1) Imatinib itself is already FDA-approved, which will fasten clinical
implementation of imatinib for treatment of vascular leak, 2) the current experience with imatinib
indicates that its use is very safe when used for short interval (days to weeks), 3) for imatinib,
biological availability is high (80-90%) and the time to maximal effect is very short (2-3h). These
characteristics perfectly match the requirements for therapies in the acute setting of the ICU. In
addition, three case-reports have confirmed the efficacy of imatinib in the treatment of pulmonary
edema thus far [50,51,Chapter 9]. In the latter (Chapter 9), we were able not only to demonstrate
a beneficial effect of imatinib on pulmonary edema, but measuring the pulmonary leak index, we
also showed that imatinib treatment was associated with a reduction in pulmonary vascular leak.
The very diverse pathophysiology of pulmonary edema in these three case-reports (pulmonary
veno-occlusive disease, bleomycin-induced lung injury and systemic capillary leak syndrome, respectively), suggests that the applicability of imatinib for vascular leak may be very broad. Yet, some limitations might complicate the clinical use of imatinib as ARG inhibitor. Long-term treatment of imatinib has been associated with peripheral edema [52] and intracranial hemorrhage [53]. Besides ARG, imatinib inhibits the related tyrosine kinase c-Abl, even at lower IC50-values [49]. Others and we have shown that c-Abl is required for a stable endothelial barrier [54, Chapter 3]. In addition, long-term inhibition of platelet-derived growth factor receptor (PDGFR), another imatinib-sensitive kinase, has been shown to impair blood vessel maturation, due to insufficient pericyte support [55]. In Chapter 3, we provide evidence that the protective effects of imatinib on the endothelial barrier predominantly result from inhibition of ARG. Yet, in our experiments not all imatinib effects could be reproduced with ARG knockdown (e.g. imatinib, but not ARG knockdown reduced the phosphorylation of AJ proteins like FAK and paxillin, unpublished data), while not all effects obtained by ARG knockdown could be reproduced by imatinib (e.g. ARG knockdown, but not long-term treatment with imatinib increased expression of α5β1 integrin, Chapter 4). Pointing toward considerable off-target effects and lack of specificity, these considerations prove imatinib as suboptimal inhibitor of ARG. These limitations drive the search for more specific ARG inhibitors, which preferentially inhibits ARG, but not c-Abl.

Currently, two alternative small molecule inhibitors are available for inhibition of ARG, nilotinib and pazopanib [49]. Nilotinib (AMN107) was developed from the chemical imatinib structure, with 20-fold more potent Abl inhibition and effective in imatinib-resistant forms of chronic myeloid leukemia [56]. Nilotinib was repeatedly shown to prevent against acute lung injury [57-59]. Although more potent than imatinib, nilotinib still carries a similar inhibitor profile. Developed for inhibition of c-Abl, nilotinib will therefore meet the same limitations as imatinib, when used as ARG inhibitor. This may not be the case with pazopanib (GW786034), which was found to have higher inhibitory activity against ARG than against c-Abl [49]. Pazopanib was developed for renal cell carcinoma, and its inhibitory profile comprises amongst others vascular endothelial growth factor (VEGF) receptor-1, -2, and -3, PDGFR-α, PDGFR-β, and c-KIT [60]. Besides its activity against ARG, this may be a suitable inhibitory profile, since VEGF is a well-known barrier disruptive agent [61] known to be elevated in the circulation of septic patients [62], and since our studies have shown that inhibition of PDGF and c-KIT improves endothelial barrier function (Chapter 3, Figure 5). Interestingly, in a recent series of publications pazopanib was shown to effectively block endothelial barrier disruption [63-65]. It remains unclear, however, whether the beneficial effect should be contributed to the inhibition of ARG, or inhibition of other kinases in the pazopanib inhibitory profile (e.g. VEGFR, PDGFR or c-KIT).
For the pharmacological inhibitors described above, ARG inhibition has emerged as off-target effect, rather than intended. As far as we know, there are no specific ARG inhibitors available (personal communication A. Koleske). The fact that ARG is relatively understudied (the PubMed search: “Abl2 OR Abl-related gene” yields a total of 138 publications), may have contributed to the lack of specific ARG inhibitors. Based on the results of this thesis, we hypothesize that specific ARG inhibitors will benefit treatment of endothelial barrier dysfunction and vascular leak.

In 2011 the crystal structure of ARG/Abl2 has become available [31]. The exact knowledge on the structure of ARG means a step forward in the development of pharmacological inhibitors. Currently collaborations are going on with Yale University (New Haven, USA) and Tubingen University (Tübingen, Germany) for the development of small molecule ARG inhibitors. This joint venture is intended to start with in silico comparison of ARG protein structure with chemical databases, which will yield information about ARG specificity of existing kinase inhibitors. The advantage of this quick initial approach is that the existing kinase inhibitor collections have been chemically characterized, and kinase data are already available. In parallel, the ARG crystal structure will be used to design specific small molecule inhibitors. Both approaches (compound screening and structure-based design) will be used for development of ARG-specific inhibitors.

2.2 – Development of clinical-grade genetic interference of ARG

While the chemical approach described above is complicated by limited specificity and unwanted off-target effects, genetic interference may target a gene/protein of interest with higher specificity. This approach involves irreversible binding of oligonucleotides to mRNA of the target gene, eventually followed by degradation of the mRNA and reduced expression of the target protein [66]. Specificity for the target of interest is achieved via an oligonucleotide sequence (for small interfering RNA usually 21-23 base-pairs), complementary to the mRNA of the target gene. In Chapter 4 we have optimized siRNA-mediated knockdown of ARG using two single siRNA sequences, leading to 90% knockdown of ARG expression on the protein level. These data show that protection against endothelial barrier disruption is better after complete protein knockdown than after imatinib pretreatment, suggesting either that ARG inhibition is incomplete after imatinib or that imatinib enhances barrier disruption by an ARG-independent effect (e.g. c-Abl inhibition). Otherwise, optimal ARG knockdown was associated with a slight reduction in basal endothelial barrier function. As imatinib pretreatment did not affect basal endothelial barrier function, this suggests that ARG has functions other than its kinase activity (e.g. as structural of adapter protein) [30], that are inhibited by genetic interference, but not after small molecule inhibition (which only targets kinase function). These differences should be taken into account when developing genetic approaches to target ARG. A drawback of particular importance in
the acute setting of sepsis and ARDS is that RNA interference needs considerable time to exert its biological effect. Depending on the protein and its half-life, it may take days before optimal knockdown of the target protein is observed, while sepsis and ARDS may present as clinical emergencies that ask for swift intervention [67]. Although these inherent drawbacks still limit clinical application of RNA therapeutics, pilot studies are ongoing to test the applicability of genetic ARG interference in mouse models of vascular leak.

2.3 – Conclusion part II

As such, the discovery of the barrier-protective effect of imatinib and the identification of ARG as novel mediator of endothelial barrier disruption has yielded a number of opportunities for therapeutic interventions in the field of vascular leak and edema. We show that imatinib is a suitable agent for treatment of endothelial barrier disruption, not only in cultured endothelial cells and animal models of vascular leak, but also in a first series of case-reports. Despite these promising results, efficacy and safety of imatinib may be limited by lack of specificity. Future plans involve the search for ARG-specific pharmacological compounds and ARG-directed genetic interference. As the relevance of vascular leak in the pathophysiology of sepsis and ARDS becomes increasingly acknowledged [68], and as immunomodulatory interventions have largely failed in this context [69], treatment of endothelial barrier dysfunction may form a promising approach to the patient with sepsis or ARDS.

PART III – RECOGNITION OF VASCULAR LEAK AND EDEMA FORMATION IN CLINICAL PRACTICE

Besides development of therapeutic interventions, understanding of endothelial barrier regulation may contribute to recognition of barrier disruption in clinical practice. In the final part of this thesis we have addressed the question whether parameters of vascular leak may contribute to the recognition of ARDS. Despite the central role of vascular leak in the pathophysiology of ARDS, current ARDS definitions have not implemented parameters of vascular leak in their diagnostic criteria [70-72].

Vascular leak due to disruption of the endothelial barrier is characterized by leakage of plasma proteins to the interstitium [73,74]. In Chapter 6 we therefore hypothesized that loss of protein from the circulation predicts vascular leak and that serum levels of albumin and transferrin (which have comparable molecular size of 67 and 80kDa, respectively) can be used as predictors for ARDS. Chapter 6 demonstrates that addition of serum protein levels to the existing clinical criteria (AECC criteria, LIS) improves the performance of these criteria when compared to the pulmonary
leak index as golden standard. This study is in line with other studies showing that plasma proteins predict ARDS severity and mortality. Although acute phase reactions may confound the relation between protein levels (since both albumin and transferrin are negative acute phase proteins because of increased consumption and decreased production), our study adds to the existing literature by showing that vascular leakage is a major determinant of the relation between protein concentration and ARDS severity/mortality.

In Chapter 7 we developed a novel approach to further identify plasma biomarkers of ARDS. Systematically reviewing literature on ARDS plasma biomarkers, we performed a meta-analysis on plasma biomarkers studied before, generating a ranking of plasma biomarkers according to strength of association with ARDS diagnosis and mortality. In this ranking, endothelial markers like Ang-2 and vWF perform relatively well. Plasma levels of Ang-2 and vWF are determined by release of Ang-2 and vWF from the Weibel-Palade bodies in endothelial cells [75], which is strongly increased after endothelial activation by barrier-disruptive agents [62,75,76]. The outcome of this meta-analysis therefore suggests a relatively strong relation between endothelial damage and ARDS diagnosis/mortality. Although future studies are required for the clinical application, this meta-analysis suggests that biomarkers, or panels of biomarkers [77,78], may boost ARDS diagnosis.

To date, a peculiar mismatch between the presence of vascular leak and ARDS diagnosis seems to exist. This mismatch is illustrated by the work of others and us. Esteban and coworkers have demonstrated a relatively poor relation between ARDS diagnosis according to the AECC criteria and post-mortem lung tissue analysis [79]. Only 75 percent of patients with presence of alveolar hyaline sheets at autopsy were diagnosed with ARDS according to the AECC criteria [79]. The presence of hyaline sheets is considered pathognomonic of ARDS [80] and thought to arise from extravasation of fibrinogen to the alveolar space [81]. Despite the study of Esteban and despite calls to include parameters of vascular leak in the ARDS diagnosis, also the newest criteria, The Berlin Definition, do not include parameters of vascular leak. After publication of the Berlin criteria, the study of Esteban and co-workers was repeated with similar methods. Like in the first study, the new Berlin ARDS criteria poorly matched autopsy findings [82]. In line with the work of Estaban et al., we show in Chapter 6 that current definitions of ARDS (AECC criteria and LIS) poorly correlate to pulmonary vascular leak, as measured by the pulmonary leak index. The data in the third part of this thesis therefore suggest that addition of biomarkers that reflect vascular leak – like albumin and transferrin (Chapter 6), but also Ang-2, vWF (Chapter 7) – to existing definitions may improve diagnostic accuracy of these definitions.

Finally in Chapter 8, we tried to find clinical parameters that predict the development of edema upon fluid loading in the critically ill. Fluid loading for hypovolemia is often associated with
edema formation [83], and it remains difficult to predict which patients do develop edema and do not. Since theoretical models have predicted that increased permeability enhances edema formation at increasing hydrostatic pressures [74], we hypothesized that patients with increased vascular permeability (as evidenced by the pulmonary leak index) will be at higher risk for edema formation upon fluid loading. Surprisingly, our data show that hemodynamic parameters like cardiac index and pulmonary blood volume index have a stronger relation with edema formation than parameters like PLI (Chapter 8). Patients at a plateau phase of pulmonary blood volume index may likely develop pulmonary congestion and subsequently edema upon fluid loading, which stresses the relevance of perfusion surface area as determinant of edema development [84]. Indeed, perfusion surface area is represented in the Starling equation – the product of surface area and hydraulic conductivity both yield $K_{fc}$ (see also General Introduction). The higher the perfusion surface area, the higher $K_{fc}$, and the higher, in turn, the net fluid passage ($J_v$).

In addition to low hydrostatic pressure difference [85], a low pulmonary blood volume index may therefore protect against edema formation in two ways: first, by reduced perfusion surface area, and second, by limiting perfusion of areas of damaged endothelium [86]. From a clinical perspective, the data presented in Chapter 8 indicate that fluid loading is safe in patients at low pulmonary blood volume index and low cardiac index.

**LIMITATIONS OF THE STUDY**

The work presented in this thesis carries a number of limitations. These limitations concern the methods used at all levels of research, in vitro, in vivo and clinical. Concerning the in vitro experiments, we predominantly used human umbilical vein endothelial cells. Although these cells are freshly isolated and well characterized with respect to their endothelial functions, the relevance of this cell type for the study of pulmonary vascular leak remains debated. To address this issue, key experiments were repeated in freshly isolated pulmonary microvascular endothelial cells. In addition, results from in vitro experiments with human umbilical vein endothelial cells were established in in vivo models of vascular leak. Second, for in vivo evaluation of vascular leak, we performed the Miles assay and the cecal ligation and puncture sepsis model. For the Miles assay the following should be taken into account: the assay is performed with a single barrier-disruptive agent (usually VEGF), and vascular leak is induced in the back skin. These issues may limit the translational value of the in vivo experiments, since clinically relevant vascular leak usually results from the increased concentration of multiple barrier-disruptive agents, sometimes even referred to as a ‘cytokine storm’. In addition, problematic vascular leak in the critically ill is usually not located in the (back) skin, but in vital organs like the lungs, the kidneys, the...
liver and the heart. These issues are addressed in the cecal ligation and puncture sepsis model, in which a large number of cytokines is increased in the circulation, and in which multi-organ failure is evident [87]. The advantages of the cecal ligation and puncture (CLP) model at the same time yield its main limitation – the high variability in outcome as previously reviewed [88].

In particular, vascular leak in the lungs was quite variable, with some CLP mice demonstrating evident pulmonary vascular leak, while others are at control level (Chapter 3, Figure 7). This may limit the use of the cecal ligation and puncture for evaluation of pulmonary vascular leak. An alternative model to study acute lung injury and pulmonary vascular leak may be the tracheal instillation of lipopolysaccharide, which was shown to give robust and reproducible vascular leak in the lungs [89].

A final limitation to be addressed here concerns the clinical studies (particularly Chapters 6 and 8), in which the same patient cohort was used for two different studies. By performing multiple statistical tests on the same cohort, the chance of false statistical significance increases. One way of circumventing this possibility is by testing the same hypothesis in various ways either by testing different parameters or by using different statistical approaches. Examples of the first include testing for both albumin and transferrin (Chapter 6), and examples of the latter include testing by both receiver-operating-curve statistics and multiple regression analyses (Chapter 8). Yet, a definite solution will be prospective validation of our findings in larger, multicenter cohorts.

**GENERAL CONCLUSION**

Altogether, this thesis shows that understanding of endothelial barrier regulation may improve treatment and diagnosis of syndromes characterized by vascular leak and edema formation. Important conclusions drawn from this work include:

1. Cell-matrix interaction is an important determinant of endothelial barrier function.
2. During endothelial barrier disruption the tyrosine kinase ARG actively regulates cell-matrix interaction, which is paralleled by intercellular gap formation.
3. Inhibition of ARG attenuates endothelial barrier disruption, which is effectively achieved by the tyrosine kinase inhibitor imatinib.
4. ARG activity is increased in the pulmonary vasculature of septic patients.
5. Imatinib is a suitable agent for treatment of endothelial barrier disruption, both *in vitro*, *in vivo* and *in clinico*.
6. Biomarkers related to endothelial barrier dysfunction (albumin, transferrin, Ang-2, vWF) strongly relate to ARDS diagnosis and mortality.
7. Clinical parameters of endothelial barrier dysfunction and vascular leak may improve diagnostic accuracy of existing ARDS definitions.
REFERENCES


