8.1 SUMMARY: THE MICROVASCULAR ENDOTHELIAL CELL IN SHOCK

Multiple organ dysfunction syndrome (MODS) is a complication of hemorrhagic (HS)- and septic shock and related to high morbidity and mortality. Interaction between activated neutrophils and activated endothelial cells is considered to play a prominent role in the pathophysiology of MODS (as introduced in CHAPTER 1). Insight in the nature and molecular basis of endothelial cell activation during shock can assist in identifying new rational targets for early therapeutic intervention and in evaluating frequently employed interventions in shock. In this thesis we used models of hemorrhagic- and septic shock to study microvascular endothelial responses in shock. We hypothesized that shock induced stress (being it sepsis or hemorrhage induced) activates microvascular endothelial cells to a pro-inflammatory state, and that microvascular beds in different organs would respond differently to the same stress. This pro-inflammatory endothelial activation can attract leukocytes to the organs, leading to organ damage. Understanding the nature, the molecular control, and the microvascular bed specific differences of these processes may allow us to identify targets for therapeutic intervention.

In CHAPTER 2, we examined the kinetics and organ specificity of endothelial cell activation in a mouse model of HS. Anesthetized mice were subjected to controlled hemorrhage to a Mean Arterial Pressure (MAP) of 30 mmHg. Mice were sacrificed after 15, 30, 60 or 90 minutes of HS. After 90 minutes of hemorrhagic shock, a group of mice was resuscitated with 6% hydroxyethyl starch. Untreated mice and sham shock mice that underwent instrumentation and 90 minutes of anesthesia without shock served as controls. Gene expression in kidney, liver, lung, brain, and heart tissue was studied. Induction of inflammatory genes occurred early during HS and already before resuscitation. Expression of adhesion molecules (P-selectin, E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1)) was significantly induced in all organs, albeit to a different extent depending on the organ. Endothelial genes CD31 and VE-cadherin, which function in endothelial cell homeostasis and integrity, were not significantly affected during the shock phase. The rapid inflammatory activation was not paralleled by induction of hypoxia-responsive genes (vascular endothelial growth factor and hypoxia-inducible factor 1α). This study demonstrated the occurrence of early and organ-specific endothelial cell activation during hemorrhagic shock.
Mechanical ventilation (MV) is frequently employed in patients with HS. Intubation and MV protects organs from hypoxia and hypercapnia, on the other hand MV itself may initiate an inflammatory reaction and induce inflammation of the lung and distant organs. So in **CHAPTER 3**, our aim was to investigate the consequences of mechanical ventilation of mice subjected to HS on microvascular endothelial activation in the lung and kidney, as well as the role of systemic hypoxia in EC (endothelial cell) activation in these organs. As already evident from chapter 2, 90 minutes after shock induction a vascular bed specific, heterogeneous pro-inflammatory endothelial activation represented by E-selectin, VCAM-1 and ICAM-1 expression was seen in kidney and lung. No differences in endothelial adhesion molecules between the spontaneous breathing and mechanically ventilated mice were found. In the presence of HS, no differences in organ mRNA levels for endothelial pro-inflammatory cytokines (TNF-α, IL-6, MCP-1) were found between the spontaneous breathing and mechanically ventilated mice. In plasma, CXCL-1 and IL-6 were increased 90 minutes after shock induction, with no differences between HS alone and HS combined with mechanical ventilation. During HS, HIF-1α mRNA was not induced in the kidney, while in the lung HS led to HIF-1α mRNA upregulation, with no differences between HS alone and HS combined with mechanical ventilation. To determine the contribution of tissue hypoxia to the observed changes due to decreased oxygen delivery to the pro-inflammatory endothelial activation during the shock period, we subsequently studied endothelial pro-inflammatory activation in response to short term exposure to severe hypoxia only. 2 hours of 6% hypoxia did not induce expression of E-selectin, VCAM-1 and ICAM-1 in the kidneys and the lung of mice. In this chapter we concluded that HS induced endothelial activation is not augmented nor prevented by mechanical ventilation during the shock phase, and that 2 hours of hypoxia alone does not lead to the endothelial activation observed in the HS model.

Adiponectin is an adipocyte-derived anti-inflammatory cytokine that has been shown to attenuate endothelial activation. Previous studies have demonstrated that sepsis is associated with reduced circulating levels of adiponectin. Thus, we hypothesized that sepsis-mediated adiponectin deficiency results in accentuated endothelial activation and secondary multi-organ dysfunction. In **CHAPTER 4**, we show that circulating levels of adiponectin are reduced in endotoxemia, but increased in cecal ligation puncture (CLP) sepsis models. Quantitative RT-PCR for adiponectin and its receptors revealed no
changes respectively significant reduction in gene expression in either model of sepsis, the pattern of response being model and organ specific. Adiponectin deficiency resulted in increased expression of endothelial adhesion and coagulation molecules in the lung, liver and kidney during sepsis, increased macrophage and neutrophil infiltration, and vascular leakage in the liver and kidney during experimental sepsis. This was accompanied by impaired survival following CLP, and soluble endothelial adhesion molecules sE-selectin and sICAM-1. These data suggest a protective role of adiponectin in diminishing endothelial dysfunction during sepsis.

In chapter 5 we reviewed the Angiopoietin/Tie2 system, which is an endothelial receptor system which influences endothelial integrity and vascular leakage, and engagement in inflammation and leukocyte recruitment (figure 8.1). The Ang/Tie system consists of the ligands Ang-1 and Ang-2, and the receptors Tie1 and Tie2. Ang-1 and Ang-2 bind to Tie2, and while Ang-1 induces Tie2 phosphorylation, Ang-2 binding competes for this binding and inhibits its phosphorylation. The receptor Tie2 is a 140 kD tyrosine

Figure 8.1. A schematic model of the Angiopoietin-Tie2(Ang-Tie2) ligand-receptor system. Quiescent endothelial cells interact with pericytes that constitutively produce Ang-1. As a vascular maintenance factor, Ang-1 reacts with the endothelial tyrosine kinase receptor Tie2. Ligand binding to the extracellular domain of Tie2 results in receptor autophosphorylation, docking of adaptors and coupling to intracellular signaling pathways. Signal transduction by Tie2 leads to vascular stabilization. Tie2 activation also inhibits adhesion molecules (for example, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin). Ang-2 is rapidly released from Weibel-Palade bodies (WPB) upon stimulation by various inflammatory agents. Ang-2 acts as a partial antagonist of Ang-1, and reduces Tie2 signaling, causing disassembly of cell-cell junctions. In inflammation, this process causes capillary leakage and facilitates transmigration of leukocytes.
Summary & Future perspectives

kinase receptor, which is shed from the EC and this sTie2 may be involved in ligand scavenging. In summary, Ang-1 can be viewed as a stabilizing messenger by continuous low level Tie2 phosphorylation and Ang-2 as a destabilizing messenger preparing the endothelium for action. The changes observed in the Ang/Tie2 system in murine and human shock and sepsis might all play a role in MODS symptoms.

One of the important organs that fail during MODS is the kidney. Both hemorrhagic shock and endotoxemia induce pronounced vascular activation in the kidney which coincides with albuminuria and glomerular barrier dysfunction. In chapter 6 we hypothesized that changes in the above mentioned Angiopoietin/Tie2 system underlie this loss of glomerular barrier function. In healthy murine and human kidney, Tie2 is heterogeneously expressed in microvascular beds. In mice subjected to hemorrhagic and septic shock, Tie2 mRNA and protein were rapidly, and temporarily, lost from the renal microvasculature, and normalized within 24 hours after initiation of the shock insult. The loss of Tie2 protein could not be attributed to shedding as both in mice and healthy volunteers subjected to endotoxemia, sTie2 levels in the systemic circulation did not change. In an attempt to identify the molecular control of Tie2, we activated glomerular endothelial cell cultures and human kidney slices in vitro with LPS or TNF-α, but did not observe any change in Tie2 mRNA levels. In parallel to the loss of Tie2 in vivo, an overt influx of neutrophils in the glomerular compartment was seen which coincided with proteinuria. As neutrophil-endothelial cell interactions may play a role in endothelial adaptation to shock, and these effects cannot be easily mimicked in vitro, we depleted neutrophils before shock induction in vivo. While this neutrophil depletion abolished proteinuria, Tie2 was not rescued, implying that Tie2 may not be a major factor controlling maintenance of the glomerular filtration barrier in this sepsis model.

To bring our pre-clinical observations to the patient we next studied the time course of Ang-2 release during human experimental endotoxemia, possible association of Ang-2 levels with the levels of soluble adhesion molecules and inflammatory cytokines, and the early time course of Ang-2 release during sepsis in critically ill patients. In chapter 7 circulating Ang-1, Ang-2, soluble Tie2 receptor, the inflammatory molecules TNF-α, IL-6, IL-8 and C-reactive protein, and the soluble endothelial adhesion molecules P-selectin, E-selectin, and ICAM-1 were measured in healthy volunteers during a 24-hour period after a single intravenous injection of LPS (4 ng/kg). In addition, the course of circulating Ang-2
was analyzed in septic patients at ICU admission and after 24 and 72 hours, respectively. During human endotoxemia, circulating Ang-2 levels were elevated, reaching peak levels 4.5 hours after LPS infusion. Ang-2 exhibited a kinetic profile similar to that of the early proinflammatory cytokines TNF-α, IL-6, and IL-8. Ang-2 levels peaked prior to the soluble endothelial-specific adhesion molecules. The increase of Ang-2 correlated with the increase of TNF-α levels, and the increase of soluble E-selectin levels. In septic patients, Ang-2 increased in non-survivors only, and was significantly higher compared with levels in survivors at baseline as well as at 24 hours and 72 hours. From this research we concluded that LPS is a triggering factor for Ang-2 release in man. A persistent increase in Ang-2 during the early course identifies septic patients with unfavorable outcome.

In summary, several studies in this thesis performed in models of hemorrhagic- and septic shock show that microvascular endothelial cells respond early in shock with a proinflammatory phenotype and that these responses are organ and vascular bed specific. This proinflammatory endothelial activation is accompanied by leukocyte recruitment and organ dysfunction. The Angiopoietin/Tie2 system is in disbalance during shock, although there is still insufficient molecular knowledge of the Angiopoietin/Tie2 system in shock states to rationally support therapeutic intervention in this system in critically ill patients (vide infra).
8.2 Future perspectives

Ever since the description of multi-organ dysfunction syndrome (MODS), patients admitted to the ICU with MODS have a high mortality irrespective whether the underlying condition is sepsis, trauma, pancreatitis, or any other major insult. Therapy has been directed at maintaining physiological parameters such as blood pressure, tissue oxygenation, glucose, and electrolytes within certain boundaries. The maintenance of physiological parameters does not treat the cause of MODS, it is merely a supportive measure.

A major setback in the treatment of MODS patients is that we do not completely understand the mechanisms underlying MODS. Immunity, inflammation, coagulation, cell death and survival, cell metabolism, vascular leakage, tissue hypoxia, and altered cell trafficking all have been connected to MODS, and all share components and represent important physiological systems, giving it the aspect of an almost intractable problem. In infection related MODS enormous efforts have been made to unravel and to intervene in the cascade of events leading from infection via MODS to death. Therapies directed at crucial points in the inflammatory and coagulation cascades have, until now, not resulted in therapeutic options that are effective in every day clinical reality.

Whether interventions failed because they are not effective or because the way these drugs are tested is inadequate, is not clear.

With the advent of promising targets for therapy aimed at microvascular dysfunction to diminish early EC activation and influencing the Angiopoietin/Tie2 signaling pathway, we have to keep the failures of the past in mind. In this chapter, we describe the route to the clinic and the hurdles that have to be overcome by focusing on intervening in the Ang/Tie2 system in shock states.

The reductionist approach and the way interventions in patients are tested

The reductionist approach attempts to describe and understand biological systems in terms of simple components and so far has revealed a multitude of important mediators of MODS. In modern drug development, compounds to influence these mediators are easily developed, and many drugs have been tested in the last decade but failed the ultimate phase III randomized controlled trials. As an example of a mediator one could
look at TNF-α. Cachexin, later called TNF-α, has long been recognized as an early response molecule in inflammation. With monoclonal antibodies and soluble TNF-α receptors this pro-inflammatory cytokine can be neutralized. In *in vitro* studies the effects created were beneficial, and in animal models of sepsis including endotoxaemia or caecal ligation and puncture (CLP), improved animal survival was observed upon TNF-α blockade. In patients, a phase II study did not show harm, and suggested a good survival with improvement of clinical chemistry supposed to predict survival. However, the ultimate test of a phase III study on anti-TNF-α antibodies did not decrease mortality. All tests prior to the randomised controlled trial (RCT) supported the fact that the drug was effective in the sense that it influenced the mediator. The reductionist approach may have overlooked important redundant systems and the complexity of the disease in a real life situation. Another possibility could be that the mediator has also beneficial functions in patients or that timing and model differences between patient and animal models can explain the failure. The functions of the mediator involved may even change during the course of the illness, for example from being vital in the initial period to detrimental later on.

The reductionist approach does not seem to be the road to take in the multi-causal, multifactorial diseases that underlie MODS. Instead a holistic or integrated perspective is needed, and as such, an RCT might not be the best way to test interventions in MODS. MODS is a syndrome instead of a disease. Trauma, hemorrhagic shock followed by an infection and insults imposed by mechanical ventilation is a usual sequence. These different insults have specific and common responses. For instance, in rat models of endotoxaemia, hemorrhagic shock and ventilator induced lung injury, these differences were elegantly unmasked by genome wide expression analysis in the injured lungs. From the more than 30,000 expressed sequences on the Affymetrix® chip, 885 genes were LPS challenge specific, 232 genes were ventilator induced lung injury specific and 301 genes were hemorrhagic shock specific, while 147 genes showed overlap between the three insults. In RCTs these insults are not standardized and in human sepsis all patients will be in a different time point in their disease. As long as we are not able in sepsis to quantify and qualify the infection and the host response, as nowadays in oncology treatment, we will not be able adequately treat these insults. In oncology for instance, gene-expression signatures are used to empower the identification of prognostic subclasses in tumor types. In early stage breast cancer the risk of relapse can be predicted by tumor gene
expression arrays. These profiles which are obtained with the use of tumor-derived mRNA assayed on microarrays are being used in clinical practice to predict prognosis and hence to influence therapeutic intervention\textsuperscript{12}. The holistic overview, taking into account interactions of different genes in damage and repair systems in MODS patients, might help to select specific subgroups of patients with this complex multifactorial syndrome, in which specific therapies are beneficial.

**Shock research: heterogenic diseases and homogeneous animal models**

Not only the sequence of insults differs, patients also differ tremendously. Patients have different life and medical histories, in addition to age, sex, and genetic background. To find out the heterogeneity of HS insults in patients treated in the University Medical Center Groningen (Groningen, The Netherlands) we performed an inventory of the different diagnoses for patients with HS (van Meurs, Nijsten et al, unpublished). We defined massive transfusion as a hallmark of HS and based on this all patients were selected who obtained a transfusion of 10 or more units of red blood cell concentrates (RBC) on a single day. The leukocytes transfused with the RBC are considered to mediate adverse effects, we therefore differentiated between patients that received leukocyte-depleted RBC (Id-RBC) and conventional RBC (c-RBC). All patients between 1-1-2000 and 31-12-2006 were identified using the local hospital and local blood bank databases. The underlying disease, the indication for transfusion, the hospital mortality, the 30 day mortality and the 1 year mortality were recorded. 510 patients received 10 or more RBCs on a single day of which 122 patients received c-RBCs and 388 received Id-RBCs. The patient categories are highly variable (table 8.1). Massive transfusion was performed during acute or elective surgery in most patients: 84% in the c-RBC group and 83% in the Id-RBC group. Within the surgical population 39% of the patients received a massive blood transfusion because of liver transplantation or cardiac surgery. The in-hospital mortality was high, 36% in the c-RBC and 28% in the Id-RBC group, and not significantly different. The challenge that we are facing is that it is highly unlikely that MODS in a liver transplant patient with HS has the same etiology and therefore similar therapeutic needs as an otherwise young and healthy poly trauma victim. Studies for markers of endothelial activation and the Ang/Tie-2 system in these patients will be hampered by these large patient differences, while patient groups are too small to select one patient
To study the effects of therapeutic interventions, RCTs are performed. In an RCT researchers stratify for known predictors of outcome. In sepsis research, researchers stratify for APACHE score and exclude patients with certain co-morbidity. For all unknown outcome determining factors, randomization is performed because of the premise that this will randomly mix these unknown factors between the groups, and therapy is the only outcome modifying factor. In MODS RCTs usually 500-3,000 patients are randomized.

### Table 8.1. Patient characteristics and mortality rates of patients transfused 10 or more conventional (c-) RBC and leukocyte-depleted (ld-) RBC units on a single day.

<table>
<thead>
<tr>
<th>Clinical context of transfusion</th>
<th>c-RBC</th>
<th>ld-RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elective surgery</td>
<td>3 (3%)</td>
<td>14 (4%)</td>
</tr>
<tr>
<td>Abdominal surgery</td>
<td>23 (19%)</td>
<td>43 (11%)</td>
</tr>
<tr>
<td>Vascular surgery</td>
<td>46 (13%)</td>
<td>40 (10%)</td>
</tr>
<tr>
<td>Liver transplantation</td>
<td>17 (14%)</td>
<td>57 (15%)</td>
</tr>
<tr>
<td>Lung transplantation</td>
<td>2 (2%)</td>
<td>30 (6%)</td>
</tr>
<tr>
<td>Cardiac surgery</td>
<td>29 (24%)</td>
<td>68 (19%)</td>
</tr>
<tr>
<td>Trauma</td>
<td>13 (11%)</td>
<td>71 (18%)</td>
</tr>
<tr>
<td>Elective medical</td>
<td>11 (9%)</td>
<td>34 (9%)</td>
</tr>
<tr>
<td>Emergency medical</td>
<td>8 (7%)</td>
<td>31 (8%)</td>
</tr>
</tbody>
</table>

| Number of RBCs (SD)           | 15.0 (6.9)| 14.3 (5.7) |
| In-hospital mortality (%)     | 44 (30%) | 111 (29%) |
| 30-day mortality (%)          | 48 (35%) | 124 (31%) |
| 1-year mortality (%)          | 61 (50%) | 195 (51%) |
| Age (years, SD)               | 57 (18)  | 53 (20)   |
| Gender (percentage male)      | 66 %     | 60 %      |

Clinical context of transfusion, age and sex of the patients and the in-hospital, 30 day – and 1 year mortality of the patients are shown in the c-RBC versus the ld-RBC group.
Summary & Future perspectives

number is determined by the large background variation in mortality and by the relatively small effect of the intervention of interest. In these large populations, patients with very different characteristics will be included due to our limited understanding of MODS. Both beneficial and detrimental outcomes of an intervention can be balanced in the study group without noticing. Post-hoc analyses can only be hypothesis generating and should always be confirmed by prospective testing\textsuperscript{13, 14}. Extensive pre-study translational reasoning and communication with medical doctors and pre-clinical researchers in the field might solve part of the problem\textsuperscript{14}. As an example of where translational reasoning could influence intervention studies in MODS, one can look at a study in which the authors demonstrated that phospho-specific whole blood flow cytometry could be used to assess activated signaling pathways in leukocytes isolated from pancreatitis patients\textsuperscript{15}. In this study pancreatitis patients showed decreased NF-κB phosphorylation in TNF-α stimulated lymphocytes, whereas phospho-p38 MAPK was increased in TNF-α stimulated lymphocytes of pancreatitis patients compared to healthy controls\textsuperscript{15}. These results from whole blood phospho-specific flow cytometry may allow for determination of immune status and provide a rational basis for timing and intensity of immunomodulatory therapies in MODS\textsuperscript{16}. In this translational way researchers and clinicians might become better in stratifying patients and diminish large inter-group variability in MODS studies. The fact that ex-vivo approaches cannot determine the microvascular endothelial cell inflammation status, as these responses are highly heterogeneous, hampers the use of this technique to stratify for endothelial inflammation.

Furthermore, RCTs in MODS patients might not be capable in identifying useful therapies and may represent a very inefficient way to try this\textsuperscript{17, 18}. In observational research, when diseases are discovered and explained, case control studies and retrospective follow-up studies might even be more valuable than RCTs\textsuperscript{19}. Cohort studies can help to define dose and timing if we can identify clinically relevant parameters for outcome\textsuperscript{4, 10}. Some of this patient heterogeneity can be taken into account when developing animal models for shock and MODS. Some heterogeneity is the result of co-morbidities and reduced physiological reserve. In the absence of significant co-morbidities, ‘healthy’ ageing still seems to be associated with increased vulnerability to insults associated with systemic inflammation, resulting in significantly worse outcomes in terms of morbidity and mortality from these insults. In our lab, for instance, Francis M. Wulfert, MD,
performed experiments in which she compared ‘healthy’ 18 months old mice with 3 months old young mice with regard to responsiveness to LPS administration (Wulfert et al, submitted). These experiments revealed that upon endotoxaemia the endothelium in aged mice responded by a more extensive increase in expression of P-selectin and E-selectin in comparison with young mice. Moreover, in aged mice circulating PMN count increased significantly upon LPS exposure, which was paralleled by an increase in PMN influx in the kidney. In the research group of Dr. I. H. Chaudry (Center for Surgical Research, University of Alabama, Birmingham, USA) the focus has been, amongst others, on sex specific differences in the immune response after major hemorrhage\textsuperscript{30}. In their rodent models female mice are protected against the detrimental effects of hemorrhagic shock. In the laboratory of our collaborator Dr. Kiichiro Yano (Center for Vascular Biology Research, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, USA) the focus is on adiposity and western style diet in relation to endothelial activation in sepsis, as previous studies suggest that obesity is associated with inferior outcome in septic patients\textsuperscript{21}. The hypothesis is that cross talk between adipocytes and endothelial cells are an important determinant of endothelial behavior in sepsis. In a mouse LPS injection model, it was found that obesity increases sepsis morbidity and mortality (figure 8.2). This increase was associated with increased endothelial activation in kidney and lung (figure 8.3).

![Figure 8.2](image_url)

**Figure 8.2. Effects of obesity on IL-6 plasma levels and mortality in endotoxemic mice.**

Control mice (CTL) (10% fatty diet fed), DIO (60% fatty diet fed) mice, were injected i.p. with 8 mg/kg LPS or with NaCl 0.9%. (A) Survival was monitored up to 96 hrs. (B) Plasma samples were obtained at 24 hrs after ip injection and IL-6 was analyzed by ELISA. All data are expressed as mean + S.D. of three independent experiments. *: p < 0.05 (van Meurs and Yano et al, unpublished).
These observed differences between mice of different sex, age and obesity will be taken into account in future studies in hemorrhagic and septic shock. Translational approaches can be used to find subgroups of patients that benefit from certain therapies, which can be researched in animal models specifically developed for this subgroup of patients.

**The road to the clinic for Ang/Tie2 based therapies**

Based on data in chapter 5, 6, and 7 one can hypothesize that the Ang/Tie2 signaling system may play a crucial role in the processes that underlie the symptoms of MODS. Since therapeutic intervention in this system has successfully passed several pre-clinical tests (summarized in chapter 5) and based on our own data from animal and human shock models (chapter 6 and 7) it is timely to start drawing a road map for further preclinical and clinical development.

To prevent the development of MODS or treat it when it has occurred, one of the key issues is to bring the endothelial cells back to a quiescent, non leakage state (chapter 5). From the various pre-clinical studies we can speculate that an increase in Tie2 phosphorylation leads to diminished inflammation and vascular leakage. This increase in Tie2 phosphorylation could be achieved by several means. First, Ang-1 and its modified products could be administered systemically, also gene therapy aimed at increasing systemic Ang-1 protein levels could be a future option\(^{22-25}\). Experimental studies with

![Figure 8.3. Effects of obesity on tissue mRNA levels of inflammatory and coagulation molecules in endotoxemic mice.](image)

**Figure 8.3. Effects of obesity on tissue mRNA levels of inflammatory and coagulation molecules in endotoxemic mice.**

Control mice (CTL) (10% fatty diet fed), DIO (60% fatty diet fed) mice, were injected i.p. with or without 8 mg/kg LPS. The results of quantitative real time PCR analyses of ICAM-1, VCAM-1, E-selectin, P-selectin, COX-2 and PAI-1 in the kidney, and lung of control treated and DIO mice. at 24 hr after LPS administration. All data was expressed as mean ± S.D. of three independent experiments. *: p<0.05, **: p<0.001, ***: p<0.0001 (van Meurs and Yano et al, unpublished).
recombinant human Ang-1 in sepsis induced MODS in mice show that rhAng-1 prevents pulmonary vascular leakage, attenuates neutrophil accumulation in lung tissue, and prevents acute kidney injury (David and Kümpers, personal communication). Experimental studies with recombinant human Ang-1 treatment of ventilator induced lung injury diminished pulmonary inflammation, VEGF, and Ang-2 expression, yet did not protect against pulmonary vascular leakage\textsuperscript{26}. As the endothelium is heterogenic between different organs and vascular beds, the effects of systemic Ang-1 administration on non leaky vessels in sepsis remain to be determined. Furthermore, this heterogeneity makes that the effects of systemic Ang-1 administration on different microvascular diseased, leaky vessels, will probably also be not homogeneous. Not only recombinant proteins can be used, also Vasculotide, a novel Tie2 agonist, is available for animal research (Dr. Dan Dumont, Sunnybrook Research Institute (SRI), University of Toronto, Canada)\textsuperscript{27}. Second, Ang-2 levels can be inhibited by humanized antibodies or fragments thereof that neutralize the activity of Ang-2. As Ang-2 is the more dynamic factor compared to Ang-1, also therapies aimed at removal of Ang-2 for instance with plasmapheresis could be effective\textsuperscript{28}. Third, modulation of co-receptors could be used to activate the phosphorylation status of Tie2, as they can influence the binding of Angiopoietins to the Tie2 receptor. Cleavage of the Tie1 receptor from endothelial cells by VEGF-A makes that Ang-1 binds with increased affinity to the Tie2 receptor\textsuperscript{29}. Furthermore, VEGF can also activate Tie2 directly, via a mechanism involving proteolytic cleavage of the associated tyrosine kinase Tie1 leading to phosphorylation of Tie2\textsuperscript{30}. Lastly, existing therapies applied to septic patients might partly exert these effects because they influence the Ang/Tie2 system. Pre-clinical data show that activated protein C induces Tie2 mRNA and protein \textit{in vitro}, and shifts the Ang-1/Ang-2 mRNA balance to favor endothelial integrity and diminish vascular leakage\textsuperscript{31}. These translational experiments might give clues for better patient selection for activated protein C therapy in sepsis, as especially subpopulations with high Ang-2 levels might benefit from therapy. To examine the effect of activated protein C in patients with the highest Ang-2 levels, the plasma from septic patients collected during the PROWESS trial (activated protein C therapy in sepsis)\textsuperscript{13}, could be analyzed for Ang-2 levels and other markers of endothelial activation, and related to outcome parameters.
Which molecular data are missing from animal models to understand the Ang/Tie2 system in shock and MODS?

There is still insufficient molecular knowledge of the Ang/Tie2 system to rationally support therapeutic intervention in this system in critically ill patients. There are several issues at a molecular level that have to be solved, and include:

1. The Tie2 and p-Tie2 distribution in the vasculature.
2. The local Ang-1/Ang-2 ratios in microvascular beds.
3. The dual agonist-antagonist function of Ang-2 in vivo.
4. The role and levels of soluble Tie2.
5. The effect of co-receptor stimulation on Tie2 phosphorylation status in vivo.
6. The effect of influencing the Ang/Tie2 system on angiogenesis and other normal physiological responses.
7. The meaning of Ang-1 levels, Ang-2 levels and sTie2 levels and their relation with p-Tie2 status in organs.

First, the receptor Tie2 itself is not equally distributed among the vascular tree. For instance, in the kidney most mRNA for Tie2 has been found in the glomerulus, while least is found in the venules (figure 6.1). Wong et al were one of the first to describe that Tie2 phosphorylation (p-Tie2), is present in all tissues using Western Blot on whole organ protein isolates. Yet, until now specific antibodies for phosphorylated Tie2 are not suitable for immunohistochemistry to study whether this p-Tie2 status is evenly distributed throughout the vasculature. Moreover, the fact that Tie2 is downregulated during shock states as presented in chapter 6, makes therapy aimed at increasing Tie2 phosphorylation status even more demanding or even impossible, as the total amount of Tie2 for phosphorylation is diminished (figure 6.2). In this thesis we tried to mimic the in vivo situation by ex-vivo incubating human kidney slices (figure 6.5) with or without sepsis mediators. Unfortunately, Tie2 was lost rapidly from the vasculature ex vivo, also when incubated without sepsis mediators. Furthermore HUVEC lost Tie2 receptor rapidly in vitro, after 3 passages the mRNA levels were already 6 fold reduced (Kurniati, Molema et al, unpublished). Also primary endothelial cells harvested from mouse kidney lost their Tie2 receptor rapidly within the first days of culture (Molema, Aird et al,
unpublished). Before we start influencing Ang/Tie2 balances in the whole body, animal studies focusing on pTie2 levels in different organs have to be performed to understand this system on an organ and vascular bed specific level in health and disease.34

Second, it is currently believed that a relative surplus of Ang-1 drives Tie2 phosphorylation, but recent experimental evidence from our own laboratory suggests that within the kidney, different Ang-1/Ang-2 mRNA ratios prevail in different microvascular segments (unpublished observations). Differences in Ang-1/Ang-2 balances cannot yet be studied on a protein level due to lack of analytical tools for quantifying mouse Ang-1 and Ang-2 protein. If the differences in mRNA reflect local protein differences, these observations imply that a resting status of the (micro)vasculature brought about by the Ang/Tie2 system is brought about by different molecular balances that depend on local conditions, and that systemic interference with the system can have spatiotemporally controlled differential effects on the microvasculature. To further understand these differences, experiments in the whole organism have to be performed, and while interfering with the Ang/Tie2 system different organ responses have to be examined. To study organ related heterogeneity, the leakage in the lungs, measured with bronchoalveolar lavage, and the leakage in the kidney, measured by albuminuria, could be compared.35

Third, recent data in HUVEC clearly show that also Ang-2 can phosphorylate Tie2 in vitro,36 suggesting that Ang-2 is a partial agonist/antagonist of Tie2 signaling,37 but we have no knowledge of the occurrence of Ang-2 mediated Tie2 phosphorylation in vivo. We also do not know the protein levels that are present in the different microvascular segments of the circulation and thereby we can not extrapolate these in vitro data to the in vivo situation. These observations warrant new studies on the effect of local Ang-1/Ang-2 balances in organs and in microvascular beds on phosphorylation status of Tie2 where neither ex vivo nor in vitro experiments are suitable to answer these important questions.

Fourth, sTie2 functions as a scavenger receptor for Ang-1 and Ang-2 in vitro, and is able to decrease p-Tie2 status in HUVEC. The role of sTie2 as a Ang-2 scavenger is not totally clear in sepsis. Recently the systemic levels of sTie2 were determined in sepsis and found to be 7.43 ng/ml whereas in post operative mostly cardiac surgical patients the levels were 5.03 ng/ml.38,39 We should be cautious about interpreting these data in a...
way as sTie2 being increased in sepsis, as the levels of sTie2 could be increased in sepsis or decreased post cardiopulmonary bypass, as found recently in our own preliminary data analysis in a large cohort of post cardiosurgical patients (van Meurs, de Vries et al, unpublished).

Fifth, there are other receptors and effector molecules that control endothelial inflammation and vascular leakage. The balances and influences of, for instance, the VEGF system and the VE-cadherin system, on the Ang/Tie2 system are not completely understood.

Sixth, the Ang/Tie2 system is involved in the control of angiogenesis and vascular stabilization. These processes are important with respect to the long term effects of intervening in the Ang/Tie2 system in sepsis. What the effect of intervention will be in patients with trauma or other diseases requiring blood vessel formation or repair deserves further attention.

Seventh, we still do not completely understand what the changes in the levels of soluble markers of the Ang/Tie2 system mean in sepsis in patients. These functions should be examined in Ang-1, Ang-2, and Tie2 conditional knock-out (k.o.) and knock-in (k.i.) mouse models. Complete knockout of Ang-1 results in lethality at embryonic day 11-12.5\textsuperscript{40}, and complete k.o. of Ang-2 results in lethality 14 days after birth in mice with a 129/J background, while in mice with a C57/Bl6 background Ang-2 k.o. leads to 10% lethality\textsuperscript{41}. Cre-Lox recombination is commonly used to generate a conditional knockout animal, in which a gene is only knocked out in a specific tissue and at a specific time, which circumvents k.o. related embryonic and early mortality. Furthermore, Cre-Lox conditional knockout mice have the advantage that redundancy pathways have less time to compensate for the functional loss of the k.o. gene than in the non conditionally k.o. animals.

Summarizing, it is still largely unknown how the Ang/Tie2 system molecularly controls microvascular function \textit{in vivo} in the local environment within the organs, and how it changes at a molecular level in critically ill patients, and what the consequences of these changes are for local microvascular behavior. Extensive research is needed to translate preclinical models to patient care. Some of the research possibilities and caveats in patient studies, LPS challenged healthy volunteers, animals and cells are discussed in

## SUMMARY & FUTURE PERSPECTIVES
this thesis. Before therapeutic intervention in patients is initiated, the spatiotemporal changes in expression of the Ang/Tie2 members, their effects on phosphorylation of Tie2 and downstream consequences in microvascular beds have to be known.

Future outlook

Optimize animal models, select organs and time points for intervention and optimize research in patients.

Differences and similarities between the animal models used and the patients presenting in the clinic have to be firmly established. Animal models have to be adapted to the clinical situation as good as possible. An excellent example of how this can be approached was given by studies in the early 2000s in the laboratory of Dr. John M. Harlan on anti-endothelial adhesion therapy. In animal models of different inflammatory diseases and shock, therapy with anti-CD18 antibodies that block leukocyte-endothelial cell interaction showed protective effects. The clinical disappointment was extensive when in hemorrhagic shock the blockade of CD18, a part of LFA-1 complex on leukocytes that affects binding to endothelial adhesion molecules ICAM-1 and VCAM-1 (see introduction), was not protective. Harlan’s lab next compared the ischemia times of Ischemia/Reperfusion (I/R) animal models and those generally observed in the clinic, and found the latter to be generally longer than in the animal models. Increasing the I/R time in an animal model in which muscle injury was induced by aortic clamping demonstrated a shift from a CD18-dependent injury at short I/R times to a CD18-independent mechanism at longer I/R times. This example shows that it is of the utmost importance to develop models that mimic the clinical time frame of diseases as well as that of therapeutic intervention. Furthermore, we study our interventions in young healthy male mice while intensivists are faced with older, often female, patients with multiple co-morbid diseases. These factors are known to have its effects on MODS complexity, yet only have been sparsely studied so far in mouse models. We might try to optimize our animal models to bring sex, age and comorbidity differences into account in these models. If pre-clinical models are optimized for therapeutic results rather than on mimicking the clinical situation, we will end up with state-of-the-art models that show impressive effects in treatment groups that have no predictive value for the human pathology.
In the case of the Ang/Tie2 system, it needs to be studied whether the components of the system in animal shock models act similar to those critically ill patients. When deviations are discovered, they have to be properly defined as well as their consequences for general physiology, pathology and pathophysiology. As it is - in general - not possible to sample human/patient organs at various time points, researchers and clinicians are dependent on measuring the soluble factors Ang-1, Ang-2 and sTie2, for instance in urine, sputum and blood in humans and in mice in healthy and diseased conditions, and relate this to organ specific changes observed in mice. The organ specific endothelial response pattern in E-selectin, P-selectin and VCAM-1, ICAM-1 expression seen in sepsis models cannot be unmasked by measuring soluble endothelial adhesion molecules, nor using skin biopsies, so the translation of soluble markers and organ function in patients to organ related differences in mice is of the utmost importance. Most medical therapies were initially developed in animal research, but only about one third of highly cited animal research from top scientific journals were successfully translated and replicated in human randomised trials.

Observations of changes in the Ang/Tie2 system have to be dissected at multiple levels, i.e., at the level of the organs and time frame of the pathology related cell dysfunction. It could very well be that the Ang/Tie2 molecular system is protective in one organ system, while it is detrimental in another one, or protective but at another time point during disease development. Focusing on the lung and the kidney, two major organs involved in MODS, may prove worthwhile as it is relatively easy to get close to the endothelium by analyzing urine and sputum both in mice and men. Moreover, technically it would be possible for these two organs to deliver drugs selectively for example via catheters in local feeding arteries. The initial step in selective drug delivery to the injured kidney in AKI have been made in rats. An experiment of mind might be to deliver Ang-1 selectively to the kidneys early in critical illness by arterial catheterization. The effect can then be monitored by urine output, proteinuria, and other markers of acute kidney injury, besides monitoring Ang/Tie2 protein accumulation, or lack thereof, in the urine. The purpose can be either protection of an organ not yet involved or repair of an organ with a dysbalanced Ang/Tie2 system and concurrent pathophysiology. This local delivery strategy could be beneficial when organs are in a different phase of dysfunction.

Furthermore, large standardized insults to patients could be used to study the Ang/
Tie2 system in patients. Insults like open heart surgery or oesophagus resection could be used to study the levels of circulating Ang-1, Ang-2, and soluble Tie2 in time and relate these levels to proteinuria, kidney failure and other mediators and markers of systemic inflammation. When we use large and well documented cohorts of patients, these analyses can be done in patients with organs at risk, for instance by using RIFE criteria for kidney injury and match other patients in this large cohort with the same risk factors but no kidney failure.

The massive amount of data, already know for many years as well as those newly generated, probably cannot be handled with the statistics that are used nowadays. Maybe even methods from astrophysics, social studies, or other disciplines that are used to handle large multi-dimensional databases have to be borrowed52.

Conclusion

Endothelial cell function is severely altered in a spatial-temporal pattern in shock states and in MODS. There is an early EC activation in brain, lung, heart, liver and kidney, with organ and vascular bed specific differences in HS and sepsis. Amongst others the Ang/Tie2 system changes its expression pattern. Intervening in the Ang/Tie2 system bears a promise for future therapy of MODS, but we need to create a better understanding of what happens with this system at the molecular, cellular, organ and whole patient level, before starting to test therapeutic interventions. Animal models have to be optimized, the way we test interventions in critically ill patients have to be redefined, and the effects we aim for in patients and animal models have to be defined. Only then can we hit this long and winding road where intensive and close communication between researchers and clinicians will help us examine the multiple levels of the complex interplay drawn above at the same time. Early translational approaches will be needed to prevent therapeutic failure in a late phase of research. Although therapeutic success of Ang/Tie2 modulating therapies is not the only imaginable benefit of this research, we will certainly learn and understand beyond this scope, about the devastating aetiology and the vascular consequences of MODS in critically ill patients. This in depth knowledge will certainly be valuable for the design of new treatment options for critically ill patients in the (near) future.
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