CHAPTER 1

GENERAL INTRODUCTION & AIM OF THE THESIS
This thesis focuses on endothelial behavior in critical illness. The research questions originated at the bedside of the critically ill shock patient, were researched in the laboratory, and eventually have to end at the bedside of the critically ill shock patients. This introduction was written to introduce the basic researcher to the critically ill shock patient and to introduce the practicing intensivist to the endothelial biomedicine research field. Interested readers from either field are referred to excellent text books on endothelial biomedicine and critical care for more in depth reviews on both topics.

1.1 General introduction

1.1.1 History of critically ill patients and the ICU

‘A sharp nose, hollow eyes, collapsed temples; the ears cold, contracted, and their lobes turned out: the skin about the forehead being rough, distended, and parched; the colour of the whole face being green, black, livid, or lead-coloured. …………. it is to be known for certain that death is at hand.’ To Hippocrates, one of the founders of western medicine, the previous sentence was a description of imminent death. For the modern intensivist the first part is an indication of shock. It denotes the start of resuscitation and other active interventions, which, hopefully, will transform imminent death into survivable critical illness.

Critically ill patients are patients that would die without the help of machines and life support measurements. From the description by Hippocrates, it can be concluded that critical illness was a syndrome recognized already by the ancient Greeks. Critical care historically evolved from the recognition that the needs of patients with acute, life-threatening illness or injury could be better treated if they were grouped into specific areas of the hospital, leading to the so called Intensive Care Units (ICUs). ICUs roots can be traced back to the Monitoring Unit of critical patients initiated by nurse Florence Nightingale. During the crimen war, when Britain, France and Turkey were at war with Russia (1854), Nightingale started these monitoring units. Before Nightingale, the lack of critical care and the high rate of infection, resulted in a high mortality rate of hospitalized soldiers, reaching as high as 40% of deaths recorded during the war. Nightingale described her set of nursing rules as a ‘critical care protocol’. She stated that ‘proper use of fresh air, light, warmth, cleanliness, quiet, and the proper selection and administration of diet
– all are at the least expense of vital power to the patient. When Nightingale left for the battlefields in Turkey, she took this ‘critical care protocol’ with her. After introducing the protocol, the mortality rate fell to 2%.

In 1947, the polio epidemic raged through Europe and the United States, leaving patients paralysed, and posing a new medical challenge, which resulted in a breakthrough in the treatment of patients dying from respiratory paralysis with mechanical ventilators. In Denmark, manual ventilation was accomplished through a tube placed in the trachea of the polio patients, as these patients with respiratory paralysis required intensive nursing care. During the 1950s, the development of mechanical ventilation led to the organization of respiratory intensive care units in many hospitals. The creation of these first surgical ICUs was a natural consequence of advancement in resuscitation and support of severely ill or traumatised patients. Leaving patients alive that would have died in previous times, critical illness itself can be seen as a iatrogenic disorder.

After the start of ICUs being a place where mechanical ventilation is applied to man, further techniques for organ support and replacement were developed. For example, haemodialysis developed by Koch during the Second World War, was adapted for acute kidney injury during the Korean war. From here, critical care has developed, with patients that could benefit from ICU interventions, specialized nurses and doctors, a well defined knowledge domain, and research on all aspects of critical care.

1.1.2 The disease: classification of shock and the definition of multiple organ dysfunction

Shock is classically defined as a systemic reduction in tissue perfusion, resulting in decreased tissue oxygen delivery. Four types of shock are being recognized:

- Hypovolemic shock is a consequence of decreased preload of fluid to the heart due to intravascular volume loss. One of the main causes of this shock is massive bleeding (hemorrhagic shock, HS).
- Distributive (vasodilatory) shock is a consequence of severely decreased vascular resistance. One of the main causes of this type of shock is sepsis (septic shock), in which the host responses to host to infection is deranged.
- Cardiogenic shock is a consequence of cardiac pump failure.
- Obstructive shock is an obstruction to the heart that leads to malfunctioning. It can result from conditions such as tension pneumothorax, cardiac tamponade,
and massive pulmonary embolism.

Often the different types of shock are present in one patient\textsuperscript{10}. As an example, patients with septic shock often have a hypovolemic component due to vomiting or diarrhoea. Regardless of the type of shock a physiologic continuum of signs and symptoms is present in every patient. Shock begins with an initiating event, such as an infection (e.g., an abdominal infection) or an injury (e.g., traumatic blood loss). This initiating event produces a systemic circulatory abnormality that may progress through several complex stages. The first stage is compensated shock which is characterized by rapid compensation for diminished tissue perfusion by various homeostatic mechanisms. The first clinical sign is tachycardia and peripheral vasoconstriction. The second stage is the shock stage, during which the compensatory mechanisms fall short and signs and symptoms of organ dysfunction appear. These signs include tachycardia, hypotension, oliguria, dyspnoea, altered mental status (restlessness, fear of dying), metabolic acidosis, and a cool and clammy skin. The third stage is characterised by organ dysfunction, in which progressive organ dysfunction leads to organ damage and, if not adequately treated, death\textsuperscript{10}.

1.1.3 Therapy of shock

Patients with all kinds of shock are treated in ICUs. Therapy for haemorrhagic shock seems rather simple - asking the surgeon to ‘plug the hole’ and adding the lost circulating blood volume. The hemorrhagic shock related mortality and morbidity in ICUs has hence shifted away from the early deaths of massive bleeding in the last century towards patients with multiple failing organs. Advancement in shock resuscitation has furthermore occurred during military conflict because of the large number of patients during war. Shock resuscitation is an intervention that has changed the epidemiology of deaths from hemorrhagic shock\textsuperscript{9}. Initially patients did not receive any resuscitation and died at the battlefield. In later years patient survived because of initial resuscitation, but they developed single organ failure, with acute renal failure being the first major problem. In later days the acute respiratory distress syndrome (ARDS) became manifest. With the introduction of intensive care units these single failing organs were supported with renal replacement therapy and mechanical ventilators. With support for the lung and the kidney patients did not die of single organ failure anymore but developed
multiple failing organs. Although the mortality of multiple organ failure is decreasing, it remains the major cause of prolonged ICU stay. Therapy for septic shock seems more difficult, as the normal host response to infection is complex. Severe sepsis is defined as sepsis with more than one sign of organ failure. Septic shock is the most severe spectrum of this continuum being defined by severe sepsis with refractory hypotension. While severity increases from sepsis to severe septic shock, the mortality also increases from 15% to 45%. The host responds in such a way that it localizes bacterial invasion and starts repair of injured tissue. This inflammatory process is normally accompanied by activation of immune cells, production of factors that become systemically available, endothelial cell activation and many other processes. Sepsis arises when the inflammatory response to infection becomes generalized and extends to involve normal tissues remote from the initial site of injury or infection. As this thesis mainly focuses on hemorrhagic shock and septic shock, the other causes of shock are not further described.

1.1.4 MODS: theories and therapies

The concomitant failure of multiple organ systems after shock was first described in 1969 by Skillmann et al. Arthur Baue placed this failure of multiple organs in its context in an editorial in which he wrote: ‘However, it seems that the major limiting factor after injury in patients who do not have brain injury is not so much a system, but rather a combination of events that can best be called multiple systems failure, progressive systems failure, or sequential systems failure’. Although not a well defined syndrome, it involves progressive failure of many or all systems after an overwhelming injury or surgery. Nowadays there are no direct therapies to reverse multiple organ dysfunction syndrome, and organ support is initiated to borrow time for the body to recover.

Patients admitted to the ICU with multi-organ dysfunction syndrome (MODS) have a high mortality. Surprisingly, this high mortality does not primarily seem to depend on whether the underlying condition is septic shock, hemorrhagic shock or another insult. After treating the underlying condition, therapy is often focused on maintaining physiological parameters such as blood pressure, oxygenation and acid-base status, glucose and electrolyte levels within certain boundaries by the application of vasopressors, dialysis, and mechanical ventilation. These measures are not intended to
treat the condition underlying MODS, so they should be viewed as supportive. Optimizing these supportive therapies can therefore only prevent mortality due to complications and provide the necessary time for recuperation.

The original hypothesis that MODS was caused by tissue hypo-perfusion and oxygen debt has been challenged. In the last 30 years several other theories on the aetiology of MODS have been postulated. Some of these theories are summarized in table 1.1.

These different theories have led to several etiologic and molecule based therapies, especially for sepsis induced MODS\textsuperscript{17}. Unfortunately, apart from early, goal-directed therapy, lung-protective ventilation, antibiotics, possibly activated protein C, and treatment with selective gut decontamination\textsuperscript{18}; \textsuperscript{19}, no specific therapy has been shown valuable so far in MODS\textsuperscript{17}. A major obstacle in treating patients with MODS is that, at present, we still do not understand the precise underlying pathophysiological mechanisms. A wide variety of individually complex systems including immunological status, inflammatory activity, coagulation, cell survival, cell metabolism, paracellular

<table>
<thead>
<tr>
<th>Pathologic Process</th>
<th>Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue Hypoxia</td>
<td>Increased lactate</td>
</tr>
<tr>
<td>Uncontrolled infection</td>
<td>Nosocomial ICU-infection</td>
</tr>
<tr>
<td>Systemic inflammation</td>
<td>Cytokines (TNF\textsubscript{\alpha}, IL-6, IL-8); Leukocytosis</td>
</tr>
<tr>
<td>Immune paralysis</td>
<td>Increased anti-inflammatory cytokines (IL-10)</td>
</tr>
<tr>
<td>Endothelial activation</td>
<td>Leukocyte adherence, increased capillary permeability</td>
</tr>
<tr>
<td>Macr vascular disturbances</td>
<td>Hypotension</td>
</tr>
<tr>
<td>Micro vascular disturbances</td>
<td>Decreased microvascular flow</td>
</tr>
<tr>
<td>Dysregulated apoptosis</td>
<td>Decreased neutrophil apoptosis</td>
</tr>
<tr>
<td>Gut-liver axis</td>
<td>Increased infection, Kupffer cell activation</td>
</tr>
<tr>
<td>Mitochondrial dysfunction</td>
<td>Cellular energetics are deranged in sepsis</td>
</tr>
<tr>
<td>Production of allarmins</td>
<td>Injured tissues and cells release endogenous danger signals</td>
</tr>
</tbody>
</table>
leakage, and cell motion have been associated with MODS. All of the above mentioned systems represent dynamic processes that are closely interconnected, making a concise description of MODS an almost intractable knot\textsuperscript{20-23}. In infection related MODS, enormous efforts have been made to find options to intervene in the cascade of events leading from infection to MODS to death. However, therapies directed at crucial points in the inflammatory and the coagulation cascades have not yet resulted in therapeutic options\textsuperscript{21};\textsuperscript{22}. Many interventions have been tried but it remains unclear whether the interventions are intrinsically ineffective, or whether the manner in which these drugs have been tested has been inadequate. With new promising therapeutic targets recently entering the stage, a truly translational research approach might lead to increased knowledge on the pathophysiological mechanisms underlying MODS, and help to prevent failure of phase III clinical trials\textsuperscript{24}. Opinion leaders in intensive care medicine already suggested to abandon the randomized controlled trial in the intensive care unit\textsuperscript{25}. Some aspects of this translational approach are discussed in the \textbf{Future Perspectives} chapter of this thesis.

1.1.5 MODS the vasculature and the endothelium

The reason to focus on blood vessels to unravel part of the pathophysiology of MODS can be found in the clinical hallmarks of the often preceding shock. In shock there is hypotension, vascular leakage and leukocyte influx into organs. All these processes are regulated or facilitated by the vasculature: hypotension by diminished vascular contraction, vascular leakage by increased vascular permeability, and leukocyte influx into organs by attraction of the cells by the activated endothelial cells.

The history of the knowledge of the vascular system and the vascular wall is paved with notable names in medicine. The theory of Galen of Pergamon (129-200 A.D.) which describes the vasculature in which veins and arteries were separated, arteries transporting air into the tissues and veins transporting blood, lasted for more than 1,000 years. William Harvey was the first to describe the circulation of the blood from arteries to veins in 1628. Using light microscopy, Malpighi next discovered the existence of capillaries which connected the arterial and the venous part of the circulation. In 1839, Theodor Schwann was the first to describe the cells that line capillaries, in later years called the endothelium. The interested reader is referred to an excellent review on the history of the discovery of the capillary wall by Hwa and Aird\textsuperscript{26}. 

15
The different parts of the circulation perform different functions in health and disease. The large arteries and veins can be seen as conduit vessels, while the smaller vasculature has more specific functions. Vasomotor tone is mainly regulated by arterioles, whereas permeability is regulated in capillaries and postcapillary venules. Leukocyte transmigration takes place in capillaries and postcapillary venules. In different organs, specific anatomical parts of the circulation have specific functions, as reviewed in.

The endothelium forms the inner cellular lining of all blood vessels and is a major regulator of vessel specific functions. Endothelial cells do not act as an inert lining of the blood vessels but as active regulators and can be seen as input-output devices. The endothelium plays an important role in many physiological functions, including in the control of vasomotor tone, leukocyte trafficking, haemostatic balance, regulation of vascular permeability, regulation of angiogenesis (figure 1.1), and innate and adaptive immunity.

**Figure 1.1. Schematic presentation of the major functions of microvascular endothelial cells.**
(A) The endothelium forms a semi-permeable barrier for the transport of substances in the blood to the underlying tissue. (B) The endothelium regulates the expression of pro- and anticoagulative substances. (C) The endothelium expresses a variety of cellular adhesion molecules to tether and activate leukocytes and facilitate leukocyte adhesion and transmigration from the blood into underlying tissue. (D) The endothelium actively engages in angiogenesis in wound healing, tumour growth, as well as in a number of physiological processes. EC, endothelial cells; PAI-1, plasminogen activator inhibitor; PSGL-1: P-selectin glycoprotein ligand; sLex, sialyl Lewis x; TFPI, Tissue Factor pathway inhibitor; TM, thrombomodulin. Modified from Griffioen and Molema, with permission.
The endothelium is involved in most, if not all, disease states, either as a primary determinant of pathophysiology or as a direct and/or indirect responder to an inciting stimulus. Clinicians rarely examine the status of this cell layer, as it is not amenable to traditional physical diagnostic manoeuvres of inspection, palpation, percussion and auscultation. From a laboratory standpoint, the endothelium sheds several factors and even cells can release from the monolayer, but local organ specific reactions cannot (at present) be unmasked in this way. The differences between endothelial cells in different places in the body, the so called endothelial heterogeneity, and the fact that endothelial cells lose their vascular bed specific phenotype when cultured ex vivo and in vitro, furthermore makes that this cell layer is difficult to investigate in humans.

There is increasing evidence that the endothelium plays a central role in the pathophysiology of shock. Endothelial cells are diverse in function and highly responsive to their extracellular environment. When exposed to certain mediators that circulate in shock states, such as lipopolysaccharide, cytokines, chemokines and growth factors, endothelial cells become activated. The activation state is manifested by enhanced vascular permeability, increased leukocyte adhesion, a shift in the haemostatic balance towards pro-coagulation, and altered regulation of vasomotor tone. This activated state is considered dysfunctional when an overactive endothelium is not capable of restoring the homeostatic state, thereby causing a detrimental effect to the host. In sepsis, endothelial activation and dysfunction are critical determinants of the host response. Sepsis has a vascular phenotype, and in mice organ specific pro-inflammatory endothelial activation can be observed. The role of the endothelium as a mediator of damage to the host can be studied in sepsis models in endothelial specific KO mice. The vascular phenotype in sepsis is mediated by pro-inflammatory endothelial signalling pathways, including the pro-inflammatory NF-κB signal transduction system. Experimental studies using an endothelial specific knock out of NF-κB support the paradigm of the essential role of the endothelium as mediator of septic multiple organ failure.

Because of its strategic localisation at the interphase of blood and organs, the endothelium represents a direct possibility of communication to the various organs in the body. Furthermore, the endothelium is an attractive therapeutic target as it has a highly variable phenotype which is amenable to therapeutic modulation with drugs.
The endothelial heterogeneity, in which endothelial reactions are different in different parts of the body, could even be used to deliver drugs at specific inflamed sites of the circulation\textsuperscript{38-40}. Noteworthy is the fact that the microvascular endothelial compartment represents a pharmacologically neglected target for therapeutic intervention in inflammatory diseases such as sepsis, as the exact effects of drugs on these cells in the organs are almost completely unknown.

1.1.6 Pro-inflammatory endothelial activation, leukocyte recruitment and vascular permeability in shock

Leukocyte recruitment is a process that should be executed in any organ upon demand in response to invading organisms or damage (figure 1.2). During MODS there is an increased leukocyte influx into organs. In almost all organs, the preferred site for leukocyte transmigration into the underlying tissue upon an inflammatory challenge is the post-capillary venule, in which the endothelial cells form tight junctions between each other\textsuperscript{27}. The dimensions of leukocytes fitting narrowly in the post-capillary venules make that both cell types physically interact, allowing efficient intercellular communication\textsuperscript{41}.

Leukocytes must engage in several sequential steps to leave the circulation. Upon an inflammatory insult, one of the first reactions of the endothelial cells is to exocytose the stored, ready-to-release contents of Weibel-Palade bodies. These include the blood coagulation factor von Willebrand factor (VWF), the adhesion molecule P-selectin, and the Tie2 antagonist angiopoietin-2\textsuperscript{42}. By this means, a rapid interaction among the activated endothelium, platelets, and neutrophils is created that facilitates leukocyte rolling. Directly afterwards, via gene transcription, the endothelium produces E-selectin, which will be located on its outer membrane to exert its function. The E-selectin interacts with sialyl-Lewis X ligands expressed on the immune cells, leading to rolling of leukocytes on the endothelium. To come to a firm arrest, blood cells must engage in additional binding to endothelial cells. This takes place via the integrin family, specifically leukocyte function–associated antigen type 1 (LFA-1) and the α4β1 integrin (also referred to as very late antigen 4, VLA-4). These integrins bind firmly to adhesion molecules of the immunoglobulin superfamily including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on the endothelial cells, due to which the leukocytes come to an arrest. Thereafter the leukocyte migrates via the transendothelial
route into the subendothelial space and into the tissue\textsuperscript{43}. Leukocyte binding to endothelial cells leads to intracellular reactions in both leukocytes (reviewed in \textsuperscript{44}) and endothelial cells (reviewed in \textsuperscript{45}). The complexity of leukocyte-endothelial binding and transmigration is beyond the scope of this introduction and will not be discussed further.

Angiopoietins (Ang1, Ang2) and Tie2 are molecules involved in the control of vascular integrity and sensitization of vascular endothelial cells to inflammatory and angiogenic stimuli. There has been increasing interest in this system which is reflected by the increasing number of research papers and reviews dealing with this system\textsuperscript{46-49}. The Ang/Tie2 system is a signalling system involved in inflammation, coagulation, immunity, cell survival, cell metabolism, cytoskeleton alterations, and cell motion\textsuperscript{20}. The current paradigm describes that Ang1 binding to Tie2 increases the phosphorylation status of the intracellular part of Tie2. The competitive binding of Ang2 to the Tie2 receptor decreases its phosphorylation status. From recent studies it can be speculated that Ang/Tie2 signalling system plays a crucial role in the symptoms of MODS, and that intervening in this system can be considered a promising approach towards treatment of MODS.

\textit{Figure 1.2. Model of endothelial leukocyte interaction in inflammation (enlargement of figure I C) adapted from von Andrian and Mackay\textsuperscript{42} and Griffioen and Molema\textsuperscript{28}.}  
Endothelial cells present adhesion molecules P-selectin and E-selectin, P-selectin binds to P-selectin glycoprotein ligand (PSLG)-1 expressed on leucocytes and E-selectin binds to sialyl-Lewis X expressed on leucocytes, leading to tethering of leucocytes by the endothelium. Thereafter, integrins on leucocytes bind firmly to adhesion molecules of the immunoglobulin superfamily (IgSF) including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on endothelial cells. Thereafter, leucocytes transmigrate towards the subendothelial tissue (not shown).
In summary, endothelial cells play a vital role in tissue homeostasis. The endothelium is highly heterogeneous in time and place in the body. Moreover, the cells exert location dictated functions, and play a central role in the pathophysiology of many (inflammatory) diseases including shock and MODS.

1.2 AIM OF THE THESIS

Based on the knowledge summarized above, we hypothesised 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 (figure 1.3). Understanding the nature and molecular control of these processes may allow us to identify targets for therapeutic intervention.

As described in part 1.1 of the introduction, multiple organ dysfunction syndrome (MODS) is a complication of hemorrhagic shock (HS) and is related to high morbidity and mortality. Interaction of activated neutrophils and endothelial cells is considered to play a prominent role in the pathophysiology of MODS. Until now there is no insight in the kinetics and the molecular basis of endothelial cell activation during the induction of HS. This insight can assist in identifying new rational targets for (early) therapeutic intervention. In CHAPTER 2, we therefore examined the kinetics and organ specificity of endothelial cell activation in a mouse model of HS followed by resuscitation.

In the CHAPTER 3 of this thesis we aimed to further study the molecular effects of mechanical ventilation on endothelial cell activation. Mechanical ventilation (MV) is frequently employed in patients with HS. Intubation and MV does protect organs from hypoxia and hypercapnia, on the other hand MV may initiate an inflammatory reaction and induce inflammation of the lung and distant organs. We also examined the role of acute systemic hypoxia which was not induced by shock, to separate the effects of shock, hypoxia and systemic inflammation on endothelial cell activation.

In clinical critical care, the number of adipose patients is increasing and obesity is an independent, ‘dose-dependent’ risk factor for sepsis morbidity and mortality. Nowadays, adipose tissue is no longer considered as a storage compartment of triglycerides only, but as a highly active metabolic organ. It produces hormones and cytokines, the so called adipokines. Adiponectin is an adipocyte-derived circulating cytokine and the
most abundant product of adipose tissue. In **CHAPTER 4**, we employed the mouse caecal ligation and puncture (CLP) model as a sepsis model, and in this study we addressed the role of adiponectine on endothelial activation/dysfunction and vascular leakage.

The Angiopoietin/Tie2 system influences inflammation, vascular leakage and leukocyte recruitment. **CHAPTER 5** describes in detail the pathophysiological mechanisms of its action in shock states. As shown in chapters 2, 3, and 4, hemorrhagic shock as well as CLP induces pronounced vascular activation in the kidney. We hypothesized that the Ang/Tie2 system might control several endothelial responses during shock states. The study presented in **CHAPTER 6** hence dealt with the role of Angiopoietin/Tie2 in MODS. We studied the effects of HS and LPS administration in mice on the Ang/Tie2 system with specific emphasis on acute kidney injury.
To bring our pre-clinical observations to the patient, we lastly studied in **CHAPTER 7** the time course of changes in circulating soluble factors of endothelial activation during human experimental endotoxemia, and human sepsis, and related these to the behaviour of the soluble factors of the Ang/Tie2 system.

In **CHAPTER 8**, we summarize the outcomes of all studies presented in this thesis and put the obtained data and new insights in perspective. The focus of the future perspectives will be on the pitfalls in the way interventions in shock and MODS research were tested in patients in the past. Furthermore we will discuss the caveat that shock and MODS are heterogenic diseases which until now are tested in homogeneous animal models. To finalize chapter 8, we draw a hypothetic roadmap for implementing endothelial cell based therapies in shock and MODS with a specific emphasis on Ang/Tie2 based therapies.
REFERENCE LIST

10. UpToDate, Wolters Kluwer Health. 1-3-2011: Ref Type: Computer Program
23. Tjardes T, Neugebauer E: Sepsis research in the next millennium: concentrate on the software rather than the hardware. Shock 2002; 17: 1-8


