Protection of the microcirculation during cardiac surgery with cardiopulmonary bypass

Nick Julius Koning

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Nick Julius KONING

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Rapporteurs:
Pierre ASFAR, Professeur d'université – Praticien Hospitalier, Centre Hospitalier Universitaire d'Angers
Matijs VAN MEURS, Maître de conférences des universités – praticien hospitalier, Universitair Medisch Centrum Groningen
Hans W.M. NIESSEN, Professeur d'université – Praticien Hospitalier, Vrije Universiteit Medisch Centrum, Amsterdam
Heleen M. OUDEMANS-VAN STRAATEN, Professeur d'université – Praticien Hospitalier, Vrije Universiteit Medisch Centrum, Amsterdam

Examinateurs:
Pierre ABRAHAM, Professeur d'université – Praticien Hospitalier, Centre Hospitalier Universitaire d'Angers
Diederik VAN DIJK, Professeur d'université – Praticien Hospitalier, Universitair Medisch Centrum Utrecht

Directeur de Thèse:
Christa BOER, Professeur d'université, Vrije Universiteit Medisch Centrum, Amsterdam
Christophe BAUFRETON, Professeur d'université – Praticien Hospitalier, Centre Hospitalier Universitaire d'Ángers

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Geerten P. VAN NIEUW AMERONGEN, Maître de conférences, Vrije Universiteit Medisch Centrum, Amsterdam

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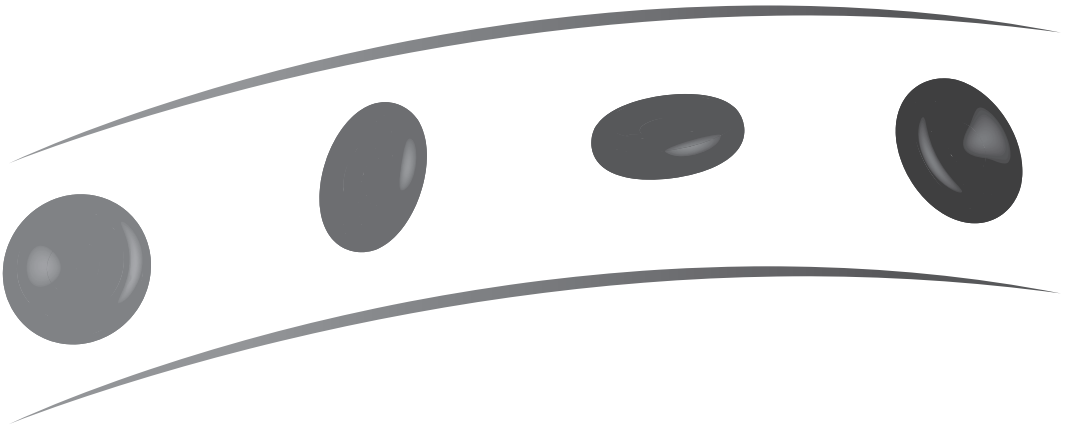
Contents

List of abbreviations

ACT	Activated clotting time
AKI	Acute kidney injury
Ang	Angiopoietin
ANOVA	Analysis of variance
AoX	Aortic clamping
BMI	Body mass index
CABG	Coronary artery bypass graft
CaO_2	Arterial oxygen content
CI	Cardiac index
CPB	Cardiopulmonary bypass
CML	N(epsilon)-(carboxymethyl)lysine
CRP	C-reactive protein
C_vO_2	Venous oxygen content
CVP	Central venous pressure
DO_2	Oxygen delivery
EBD	Evans blue dye
ECIS	Electric cell-substrate impedance sensing
HbO_2	Oxygenated hemoglobin
Ht	Hematocrit
HUVEC	Human ubilical vein endothelial cells
IABP	Intra-aortic balloon pump
ICAM	Intercellular cell adhesion molecule
ICU	Intensive care unit
IL	Interleukin
IQR	Interquartile range
MAP	Mean arterial pressure
MCP	Monocyte chemotactic protein
MFI	Microcirculatory flow index
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NGAL	Neutrophil gelatinase-associated lipocalin
NIRS	Near infrared spectroscopy
O2ER	Oxygen extraction ration
OPCAB	Off-pump beating heart coronary artery bypass
PBR	Perfused boundary region
PFP	Platelet free plasma

PPV	Proportion perfused vessels
PVD	Perfused vessel density
Rb	Cell-cell integrity
RBC	Red blood cell
RM	Repeated measures ANOVA
SDF	Sidestream dark field
SIRS	Systemic inflammatory response syndrome
TOI	Tissue oxygenation index
TNF	Tumor necrosis factor
TVD	Total vessel density
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VO_2	Oxygen consumption
vWF	Von Willebrand factor



CHAPTER 1

General introduction

Introduction

Cardiac surgical procedures are considered as high-risk operations with estimated postoperative in-hospital mortality rates of 1-2% for coronary artery bypass graft procedures to 3-6% for all combined cardiac surgical procedures [1-4], and even higher mortality rates in high-risk populations [5-6]. The underlying reason is that cardiac surgery comprises an invasive procedure, with risk for major bleeding, postoperative cardiac dysfunction or organ failure, and is mainly performed with use of cardiopulmonary bypass. Cardiopulmonary bypass is an artificial extracorporeal system, which takes over the function of heart and lungs during cardiac arrest induced for heart surgery.

Predictors of postoperative mortality following cardiac surgery, include age, gender, cardiac function, preoperative comorbidity and type of surgical procedure [3]. Additionally, intraoperative and postoperative factors may significantly alter the risk of in-hospital mortality, including cardiopulmonary bypass duration, hemodilution, blood transfusion and postoperative renal failure [7-11]. With the current increase in age and comorbidity of cardiac surgical patients, the burden of unfavorable postoperative outcome is expected to increase. As a consequence, novel treatment modalities that may reduce perioperative risk should be investigated in order to ensure low postoperative morbidity and mortality rates for these high-risk populations. It has been shown that perfusion disorders in microvessels are associated with postoperative organ dysfunction in non-cardiac surgical patients [12]. The current thesis focuses on microvascular dysfunction during cardiac surgery with cardiopulmonary bypass. We aimed to investigate mechanisms underlying disturbed perfusion of capillaries during on-pump cardiac surgery, as described below. Moreover, two treatment strategies to improve capillary perfusion following onset of cardiopulmonary bypass are investigated.

Cardiopulmonary bypass

Cardiopulmonary bypass is used in the majority of cardiac surgical procedures to ensure systemic perfusion and oxygenation during cardioplegic arrest necessary for surgical interventions. The extracorporeal system used for cardiopulmonary bypass usually consists of a reservoir collecting venous blood by gravity draining, a centrifugal or roller blood pump, an oxygenator, a heat exchanger, an arterial filter and connective tubing. The exposure of blood to the artificial surfaces of the cardiopulmonary bypass system induces a systemic inflammatory response with activation of neutrophils, the complement system, the extrinsic coagulation and production of reactive oxygen species [13,14]. Efforts have been made to improve biocompatibility of the circuits in order to reduce the inflammatory response, including coating of the extracorporeal circuit with heparin and use of closed venous reservoirs for diminishing blood-air contact [15]. However, a significant inflammatory activation is still present after onset of cardiopulmonary bypass, irrespective of preventive measures [13].

Although the use of cardiopulmonary bypass supported a rapid development in the field of cardiac surgery, its use is also a risk factor for early postoperative morbidity [16]. It has been well established that coronary artery bypass grafting (CABG) with cardiopulmonary bypass is associated with higher rates of acute kidney injury, respiratory failure, and hemorrhage as compared to CABG surgery without cardiopulmonary bypass [17,18]. However, no significant difference in mortality at 30 days or 1 year following surgery could be detected between CABG surgery with or without cardiopulmonary bypass, possibly due to the reduced graft patency following off-pump CABG surgery [17,19,20]. Research therefore focuses on optimization of cardiopulmonary bypass modalities, including limited hemodilution, minimized circuits and reduction of hemolysis.

As stated before, the systemic inflammatory response that is induced upon the onset of cardiopulmonary bypass contributes to the development of postoperative organ dysfunction, including acute kidney injury [21], acute respiratory distress syndrome [22] neurologic dysfunction [23] and bleeding disorders [24]. In previous years, most therapeutic approaches to reduce the inflammatory burden following cardiac surgery were focused on the systemic response. In addition to the use of coated systems and circuit minimization, steroid administration has been suggested to suppress the inflammatory response during on-pump cardiac surgery. Recently, two large randomized controlled trials evaluating the use of steroids during cardiac surgery with cardiopulmonary bypass have however generated contradictory results [25,26]. Although both trials failed to demonstrate a benefit of steroid administration on in-hospital mortality, postoperative respiratory failure was lower with dexamethasone treatment in the Dexamethasone for Cardiac Surgery (DECS) trial [25]. Moreover, a post-hoc analysis of the DECS-trial suggested a possible reduction of the incidence of acute kidney injury requiring renal replacement therapy following dexamethasone treatment [27]. However, the Steroids In caRdiac Surgery (SIRS) trial including high-risk patients only showed no beneficial effect of steroids on respiratory failure [26]. It has indeed been shown that administration of dexamethasone does not completely block the inflammatory response, as complement activation persists despite steroid treatment [28]. Similarly, many previous interventions aiming to decrease the systemic inflammatory response were unable to improve clinical outcome [29]. It may therefore be hypothesized that solely anti-inflammatory medication is insufficient to improve perioperative outcome following on-pump cardiac surgery. Additional factors may play an important role in explaining the morbidity associated with use of cardiopulmonary bypass during cardiac surgery.

Microcirculation

One of the additional factors causing organ dysfunction following cardiac surgery with cardiopulmonary bypass may be impaired tissue perfusion due to microcirculatory failure. The microcirculation consists of a network of arterioles, capillaries and venules. It facilitates

the transport of oxygen and nutrients to the tissues and the removal of metabolites from the tissues [30]. Adequate functioning of the microcirculation is therefore essential for maintenance of organ perfusion, and consequently for organ function.

For a long time, research on the perfusion of the microcirculation was confined to animal studies, since there were no methods available for measuring microvascular perfusion in humans. Accordingly, the microcirculation has remained concealed for the clinician. The recent development of microvascular measurement techniques in humans has renewed the interest of clinicians for the microcirculation [31]. Microvascular perfusion has particularly been studied in patients with severe inflammatory conditions, in which perfusion of the microcirculation is severely impaired [32]. Subsequently, the importance of microcirculatory perfusion disorders in the development of acute organ dysfunction is becoming increasingly apparent [33,34]. In these studies it was shown that microcirculatory perfusion abnormalities were predictive of survival in septic patients admitted to the hospital [35,36]. Moreover, these microcirculatory abnormalities were further associated with an increased number of complications in patients following major abdominal surgery [37].

In cardiac surgery, it became apparent that perfusion of the microcirculation was impaired following onset of cardiopulmonary bypass, and this impairment persists postoperatively [38,39]. Interestingly, most studies investigating microcirculatory perfusion in critical ill patients concluded that the microvasculature behaves relatively independently from macrocirculatory parameters such as mean arterial pressure or cardiac index, in particular in inflammatory conditions [38,40]. Additionally, studies investigating the use of vasoconstrictors or vasodilators were unable to improve the perfusion of the microcirculation during on-pump cardiac surgery, suggesting that the cause of impaired microvascular perfusion is more complex than merely the vascular tone of the supplying vessel [41,42]. The mechanisms behind disturbed microvascular perfusion during cardiopulmonary bypass however remained to be elucidated.

Endothelium

An important component of the microcirculation is the endothelial cell. Endothelial cells form the inner layer of blood vessels and are highly multi-functional cells involved in the regulation of local blood flow, hemostasis, inflammation, host defense and additional organ-specific functions [43]. In an inactivated state, the endothelial cells create the barrier between blood and tissue. The vessels secrete anticoagulant and vasodilatory substances through the endothelium. However, upon activation by inflammatory, infectious or thrombotic conditions, endothelial cells display adhesion molecules for blood components, induce barrier dysfunction by cellular constriction and may secrete prothrombotic agents [44]. Although this response is the result of a natural selection for coping with mostly local effects of bleeding and infection, in cardiac surgery with cardiopulmonary bypass it may be counterproductive and interfere

with perfusion of the microcirculation. In particular, endothelial barrier dysfunction may reduce capillary perfusion due to extravasation of blood plasma and increased extravascular pressure [45]. Although to date no specific treatments for promoting endothelial functioning during cardiopulmonary bypass have been described, it has been demonstrated that the tyrosine kinase inhibitor imatinib reduces endothelial barrier dysfunction in inflammatory conditions [46]. Endothelial barrier dysfunction predisposes to fluid overload following cardiac surgery, which is associated with poor clinical outcome [47]. It is currently however unclear whether reversal of endothelial barrier dysfunction may decrease fluid overload, improve microvascular perfusion and reduce organ dysfunction.

Endothelial cells are flow-sensitive cells and blood flow is therefore required for normal endothelial cell function. Shear stress on the luminal surface of the cell leads to increased nitric oxide production by endothelial nitric oxide synthase, which has vasodilating and anticoagulant properties [48]. Decreased perfusion of the microcirculation may therefore induce a vicious circle of hypoperfusion and endothelial cell dysfunction. The conventionally used nonpulsatile flow during cardiopulmonary bypass reduces endothelial shear stress and decreases nitric oxide production as compared to pulsatile flow [49], suggesting that endothelial function may be deteriorated by the loss of pulsatile blood flow during cardiopulmonary bypass. Older clinical trials demonstrate that pulsatile flow reduces mortality as compared to nonpulsatile flow during cardiopulmonary bypass [50,51], but this has not been confirmed in contemporary trials. The use of pulsatile flow during cardiopulmonary bypass therefore remains subject of discussion [52]. Moreover, whether pulsatile flow during cardiopulmonary bypass is capable to preserve microvascular perfusion is currently unknown.

On top of the endothelium resides the glycocalyx. The endothelial glycocalyx consists of a layer of large molecules covering the luminal endothelial cell surface, which contributes to mechanical transduction of shear stress to the endothelial cell, to maintenance of vascular barrier function and inhibition of leukocyte or platelet adhesion to the vascular wall [53]. The fragility of this molecular layer has complicated its investigation, as the molecules are shedded from the endothelial surface upon stimuli as inflammation, surgery, trauma, hyperglycemia or hypervolemia [54,55]. Moreover, many studies based on endothelial cell cultures ignore the presence of the endothelial glycocalyx and its functions in vivo. However, it has been shown that cardiac surgery leads to significant elevation of plasma levels of glycocalyx components, suggesting that the glycocalyx is damaged during cardiac surgery [56]. Interestingly, this elevation was more pronounced in patients undergoing on-pump surgery than in patients undergoing off-pump surgery [55]. Although it has been shown that disruption of the endothelial glycocalyx may lead to perfusion disorders in animal studies [57], the role of endothelial glycocalyx shedding for microvascular perfusion impairment and organ dysfunction following cardiac surgery remains to be investigated [58].

Blood components

Several blood characteristics may influence the perfusion of the microcirculation. The most important may be the relative presence of cells in blood plasma, so-called hematocrit. First, hematocrit is essential for tissue oxygenation because of both convective (its oxygen-carrying effect) and diffusive reasons (the effect on maintenance of capillary perfusion). A sufficient high red blood cell content in blood is necessary to maintain the number of perfused capillaries in the microcirculation and conversely, extreme hemodilution leads to a reduction in perfused capillaries [59]. This is attributed to a reduction in intracapillary pressure through reduced blood viscosity during hemodilution [60]. Additionally, hematocrit in microvessels is lower than systemic hematocrit, due to the preferential distribution of red blood cells to the high-velocity center of microvessels (Fahraeus effect) and a heterogeneous distribution of hematocrit at microvascular bifurcations (network Fahraeus effect) [61]. Hematocrit is lowered during cardiopulmonary bypass. These phenomena may add to the reduction in capillary density when systemic hemodilution is present, as occurs during cardiopulmonary bypass. Finally, red blood cells have an important function as a sensor for distribution of blood flow within the microcirculation, which may be disturbed during hemodilution [62]. From the above, it seems that a reduction in red blood cells as occurs during cardiopulmonary bypass may contribute to impaired microvascular perfusion during on-pump cardiac surgery.

Leucocytes and thrombocytes may adhere to the endothelium under inflammatory conditions, leading to capillary obstruction and increased vascular leakage, both of which may interfere with capillary perfusion [63,64]. However, the contribution of leukocytes and platelets to microcirculatory disturbances during cardiopulmonary bypass is currently unrevealed.

Rat cardiopulmonary bypass model

The role of the endothelium and the level of hemodilution in microcirculatory perfusion disturbances during cardiac surgery can only be limitedly studied in the clinical setting. The use of animal models may promote the discovery of novel physiological concepts that underlie microvascular dysfunction in cardiac surgery.

Originally, large animal models were used to study the effects of cardiopulmonary bypass on organ function. Since large animal models were highly resource demanding, multiple small animal (rat) models for cardiopulmonary bypass have been [64,65]. Initially, rat cardiopulmonary bypass models had multiple drawbacks, including relatively large cardiopulmonary bypass system priming volumes requiring blood priming, low blood flow rates, or arterial cannulation through the left ventricular apex. Subsequent studies using low volume extracorporeal systems and peripheral cannulation through the jugular vein and the femoral or caudal artery provided feasible models with the possibility of postoperative survival [66,67]. The available studies have mainly focused on inflammation and neurologic dysfunction following cardiopulmonary bypass. Rat cardiopulmonary bypass models for the

investigation of microcirculatory perfusion have so far not been described.

The rat model for cardiopulmonary bypass as developed in the departments of Anesthesiology and Cardio-thoracic Surgery of VU University Medical Center provides an excellent opportunity to improve our understanding of the mechanisms behind microvascular perfusion disturbances during cardiopulmonary bypass, and allows for testing of novel strategies to preserve microvascular perfusion. For the current thesis, we combined a rat cardiopulmonary bypass model with intravital microscopy of the cremaster muscle for study of the microcirculation (Figure 1) [67,68].

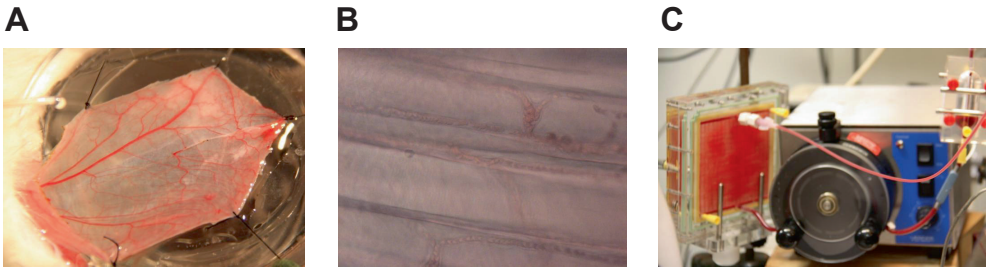


Figure 1 Cremaster muscle preparation for intravital microscopy (Panel A), example of cremaster muscle capillaries as observed by intravital microscopy (Panel B) and rat cardiopulmonary bypass system (Panel C).

Hypothesis, aim and outline of the thesis

The hypothesis in the current thesis is that endothelial cell dysfunction plays a pivotal role in the microcirculatory impairment induced by onset of cardiopulmonary bypass through vascular leakage and increased external pressure on the capillaries (Figure 2).

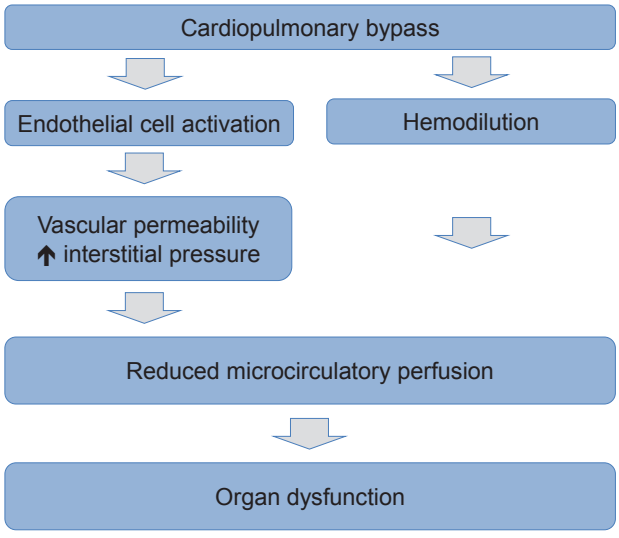


Figure 2 Central hypothesis in the current thesis.

Consequently, processes interfering with endothelial cell physiology, as nonpulsatile blood flow, endothelial glycocalyx disruption and leukocyte-endothelial interactions will lead to microvascular impairment during cardiopulmonary bypass. Moreover, hemodilution is a contributing factor to microcirculatory dysfunction. It was hypothesized that use of pulsatile flow as compared to nonpulsatile flow during cardiopulmonary bypass and that reduction of vascular leakage both lead to preservation of microcirculatory perfusion during and after cardiopulmonary bypass.
This thesis combines clinical and animal studies that aimed to investigate the mechanisms underlying microcirculatory dysfunction in cardiac surgery with cardiopulmonary bypass, in particular the role of the endothelium, the endothelial glycocalyx and the blood composition. Moreover, we aimed to evaluate two treatments strategies for preservation of microcirculatory perfusion during cardiopulmonary bypass: the use of pulsatile flow as compared to the conventional nonpulsatile flow during cardiopulmonary bypass and treatment with imatinib in order to reduce vascular leakage by inhibiting endothelial barrier dysfunction.

In **chapter 2**, we provide a review on the alterations in the microcirculation observed during cardiac surgery and the underlying factors. Moreover, microcirculatory monitoring techniques are described.
In **chapter 3**, microcirculatory perfusion disturbances are investigated in patients undergoing cardiac surgery with or without use of cardiopulmonary bypass.
In **chapter 4**, we investigate the effects of pulsatile flow versus nonpulsatile flow during cardiopulmonary bypass on the perfusion of the microcirculation.
In **chapter 5**, we describe the microvascular blood flow characteristics and related oxygenation parameters in patients during cardiac surgery with and without cardiopulmonary bypass.
In **chapter 6**, we investigate the effects of plasma from patients undergoing pulsatile or nonpulsatile cardiopulmonary bypass on *in vitro* endothelial cell barrier function.
In **chapter 7**, we describe the consequences of cardiac surgery without cardiopulmonary bypass and with nonpulsatile or pulsatile cardiopulmonary bypass on the endothelial glycocalyx using a non-invasive measurement of endothelial glycocalyx parameters.
In **chapter 8**, we study the effect of hemodilution on the reduction of cardiopulmonary bypass-induced microcirculatory perfusion disturbances in a rat model for cardiopulmonary bypass.
In **chapter 9**, we investigate the effect of imatinib as an inhibitor of vascular leakage in order to preserve microcirculatory perfusion in a rat model for cardiopulmonary bypass.

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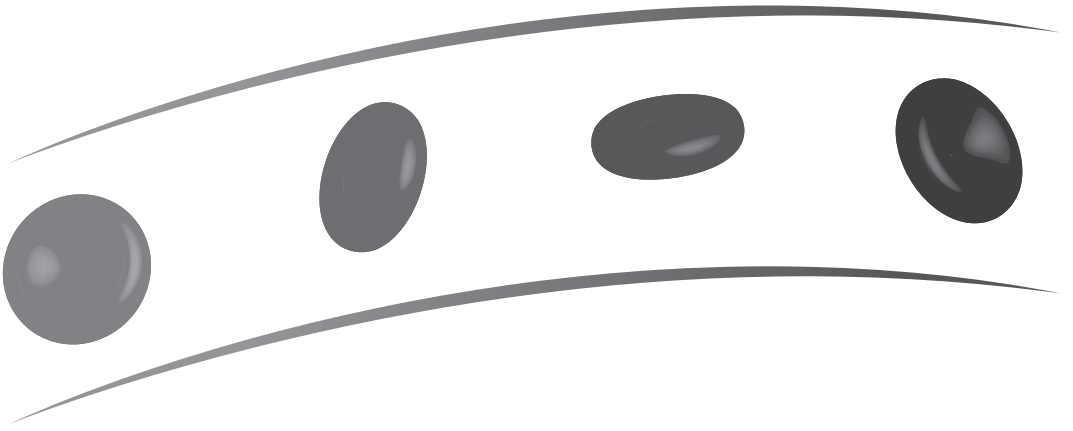

CHAPTER 2

Changes in microcirculatory perfusion and oxygenation during on-pump and off-pump cardiac surgery

N.J. Koning, B. Atasever, A.B.A. Vonk, C. Boer

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Introduction

Maintenance of microcirculatory perfusion and oxygenation is a prerequisite for preservation of organ function. Studies in the nineties already appointed gastric mucosal pH, which reflects the level of microcirculatory oxygenation, as clinical diagnostic for the level of diseases severity in critically ill patients [1,2]. This pathophysiological concept was adopted in many studies focusing on acute and chronic microcirculatory derangements in the intensive care setting [3]. The acknowledgement of microcirculatory perfusion derangements as part of the pathophysiology underlying sepsis, has led to extension of microcirculation studies to the cardiosurgical setting, as this may reflect the impact of anesthesia, surgery and cardiovascular complications on patient outcome [4,5].

In addition to the general impact of cardiac surgery on system hemodynamics, the use of cardiopulmonary bypass (CPB) is additionally associated with a wide range of changes in microcirculatory perfusion and oxygenation. Although off-pump cardiac surgery is considered to be less detrimental for microcirculatory perfusion and oxygenation than surgery with cardiopulmonary bypass, positioning of the contracting heart during off-pump procedures may also influence the perfusion and oxygenation of the microvasculature.

With the introduction of non-invasive local or regional microcirculatory monitoring techniques to assess microvascular function and dysfunction, including sidestream dark field imaging and reflectance spectrophotometry, more insight has been gained in microvascular changes during and after cardiac surgery. This review discusses alterations in microcirculatory perfusion and oxygenation during cardiac surgery with or without cardiopulmonary bypass in human subjects using these local and regional monitoring techniques. We particularly describe the influence of perioperative factors on microcirculatory integrity, such as hemodynamic and metabolic parameters, hemodilution, hypothermia, hyperoxia, nonpulsatile flow and cardiac displacement.

Microcirculatory perfusion and oxygenation

The microcirculation constitutes of resistance arteries and arterioles (10-450 µm), capillaries (5-14 µm) and venules (8-100 µm), which are covered with endothelial cells secreting anticoagulant and vasodilatory substances. The microcirculation is particularly involved in the exchange of oxygen and carbon dioxide, nutrients and metabolites between blood and surrounding tissue [6]. In a state of stress, such as hemodilution or inflammation, the endothelium presents its adhesion molecules on the blood-side surface. Consequently, activated endothelial cells lead to a procoagulant, vasoconstrictive state, in which cellular blood components may adhere to the vascular wall leading to microvascular obstruction [7]. A well-known example of microvascular obstruction is the no-reflow phenomenon following coronary interventions, which is multifactorial by nature and involves, among others, endothelial activation, inflammation, microvascular spasm, tissue edema and thrombus formation [8]. Perfusion abnormalities following microcirculatory obstruction may finally lead to reduced hemoglobin oxygenation and oxygen delivery.

Total blood flow delivery to the microcirculation is regulated by a combination of the pressure gradient over the vascular bed, the myogenic response and resistance to flow as defined by resistance arteries and arterioles [9]. In critically ill patients it has been shown that alterations in the balance between vasoconstrictors and vasodilators, proinflammatory parameters and the formation of microthrombi all contribute to a reduction in microcirculatory pressure regulation and perfusion, leading to impaired tissue oxygenation [10,11].

In addition to perfusion pressure and microcirculatory flow, local tissue oxygenation is further influenced by the countercurrent exchange of oxygen between crossing arterioles, venules and capillaries [12]. Moreover, rhythmic flow variations allow for increased oxygen offloading at the venous side of the capillaries during high red blood cell velocities, whereas oxygen would be mainly extracted by cells at the arterial side of the capillary at low red blood cell velocities [7,13]. In case of absent vasomotion during a compromised circulatory state, cells at the venous end of the capillaries would consequently be exposed to oxygen depleted erythrocytes.

The mechanism underlying oxygen delivery is often considered as a combination of diffusive determinants (surface area available for oxygen exchange or perfused capillary density, diffusion gradient) and convective determinants (hematocrit, blood flow). The diffusive determinant includes the passive movement of oxygen down its concentration gradient across tissue barriers, while the convective determinant refers to processes that generate and modulate flow in the macrocirculation or locally through paracrine signaling [14]. Both diffusive and convective mechanisms of oxygen transportation may be disturbed in cardiac surgery. Figure 1 illustrates different microcirculatory perfusion profiles under normal circumstances (panel A) and in case of a reduced diffusive and convective capacity due to

arteriolar constriction (panel B), a drop in convective capacity in case of low hemoglobin oxygenation or hematocrit (panel C) or a decreased diffusive capacity due to microcirculatory perfusion derangements (panel D).

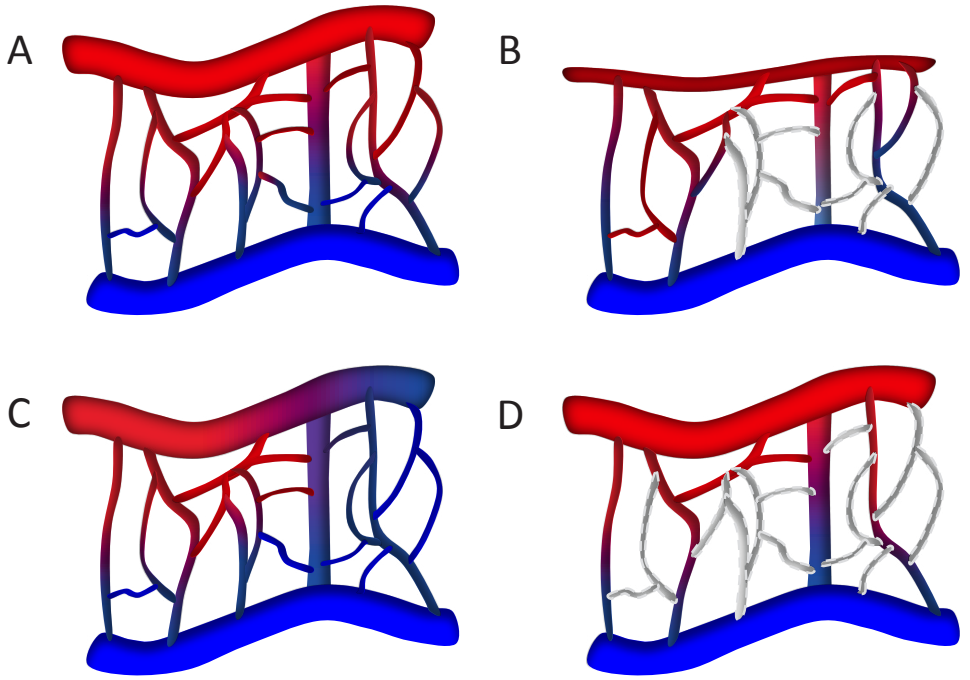

Figure 1 Microcirculatory perfusion profiles. Normal, healthy microcirculation (panel A), diminished diffusive and convective capacity due to arteriolar constriction (panel B), reduction in the convective capacity in case of low hemoglobin oxygenation or hematocrit (panel C) or a decreased diffusive capacity due to microcirculatory perfusion derangements (panel D). Red = oxygenated blood; blue = non-oxygenated blood; grey = nonperfused vessels.

Perioperative monitoring of microcirculatory function during cardiac surgery

There are several techniques available for the clinical evaluation of microcirculatory changes in perfusion and oxygenation. In particular, three-dimensional imaging techniques like positron emission tomography, somatic tissue oxygenation measurements, contrast-enhanced ultrasound and regional cerebral tissue oxygenation measurements by near-infrared spectroscopy (NIRS) are generally integrated in routine clinical practice [15]. In addition, the availability of Sidestream Dark Field (SDF) imaging and reflectance spectrophotometry increased our ability to visualize local microcirculatory perfusion and oxygenation changes during surgery [16,17].

Measurement location

Table 1 gives an overview of available local or regional microvascular measurements techniques that can be used in the cardiosurgical setting.

SDF imaging, and its predecessor Orthogonal Polarization Spectral imaging (OPS), is a technique to study human sublingual mucosal microcirculation [18,19]. The sublingual microcirculation is the most commonly used location for SDF imaging, although this microvasculature may not always reflect microvascular alterations in other, more vital organs [20]. Others however showed that, despite the distance of the sublingual circulation to the heart and central circulation, the sublingual microcirculation is a well-established site to investigate the effects of disease and therapy on microvascular function [21,22]. Moreover, changes in sublingual microcirculatory perfusion are well correlated with alterations in gastric and intestinal beds [21,22]. Alternatively, the rectal microcirculation has recently been proposed as measurement site that is more closely related to the gastrointestinal circulation [23].

Reflectance spectrophotometry measures the mucosal blood oxygenation in the terminal network of the microcirculation, but this technique has only scarcely been described for perioperative sublingual microvascular evaluation [24-26]. Alternatively, Fournell et al. used gastric reflectance spectrophotometry to measure alterations in microvascular oxygen saturation during cardiopulmonary bypass [27]. In addition to the local measurement dimensions of reflectance spectrophotometry, near-infrared spectroscopy can be used to assess regional tissue oxygenation. NIRS penetrates deeper cerebral or muscular tissue layers, and is routinely used to monitor cerebral oxygenation during cardiopulmonary bypass. NIRS is the most frequently used microcirculatory oxygenation assessment technique during surgical procedures, in particular when decreases in cerebral oxygenation are expected. The cerebral application of NIRS in cardiac surgery for neurocognitive monitoring has extensively reviewed by others and is beyond the scope of this review [28-29].

Table 1 Available techniques for local or regional evaluation of microcirculatory perfusion and oxygenation during cardiac surgery.

Microcirculatory perfusion			
Name	**Technology**	**Tissue**	**Dimension**
OPS	Orthogonal polarization spectral imaging videoscopy	Sublingual, rectal	Microcirculatory network
SDF	Sidestream Dark Field imaging videoscopy	Sublingual, rectal	Microcirculatory network
Nailfold capillaryscopy	Microvideoscopy	Skin	Microcirculatory network
Microcirculatory oxygenation			
Name	**Technology**	**Tissue**	**Dimension**
NIRS	Near-infrared spectroscopy	Cerebral, somatic	Regional
O2C	Hemoglobin oxygenation using reflectance spectrophotometry	Sublingual, gastric	Regional
Laser Doppler flowmetry / plethysmography	Microcirculatory blood flow	Skin	Regional
Tonometry / capnography	pH or pCO_2 measurements using a microelectrode	Gastric, rectal, sublingual, skin	Local
Micro pO_2	pO_2 measurements using a microelectrode	Skin	Local
Laser speckle imaging	Dynamic speckle pattern analysis during laser light illumination	Skin	Local

Sublingual, skin, gastric or rectal capnometry or oxymetry using microelectrodes could alternatively be used to assess local changes in the partial pressure of oxygen or carbon dioxide [21,30,31]. However, these techniques are still under development and not yet available for routine clinical assessment of microvascular changes during cardiac surgery. Novel techniques, like skin near-infrared laser speckle imaging, should be further evaluated to assess the feasibility of its application in the clinical setting [32].

Sidestream dark field imaging

Sidestream dark field imaging is the most frequently used optical modality for visualization of microcirculatory perfusion. This technique is incorporated in a hand-held microscope containing a light guide and a magnifying lens [18,19]. Illumination is provided by surrounding a central light guide with concentrically placed green light-emitting diodes to provide sidestream dark field illumination. The lens system located in the core of the light guide is optically isolated from the illuminating outer ring, thereby preventing the microcirculatory image from contamination by tissue surface reflections. Light from the illuminating outer ring of the SDF probe penetrates tissue and subsequently illuminates tissue-embedded microcirculation by scattering [18,19]. This leads to images where red blood cells are

depicted as dark moving globules against a bright background. To improve the imaging of moving structures, such as flowing red blood cells, the light-emitting diodes provide pulsed illumination in synchrony with the recording frame rate. This stroboscopic imaging partially prevents smearing of moving features, such as flowing red blood cells, and motion-induced blurring of capillaries due to the short illumination intervals. The advantages of SDF over OPS have extensively been reviewed by Ince et al. [33].

Reflectance spectrophotometry

Reflectance spectrophotometry ("oxygen to see"; O2C) measures microcirculatory blood oxygen saturation and hemoglobin content [34,35]. This technique illuminates tissue with visible white light. Analysis of the spectrum of backscattered light enables the calculation of the tissue optical absorption spectrum. The O2C device determines the hemoglobin oxygen saturation (HbO_2) based on differentiating absorption spectra of oxygenated and deoxygenated hemoglobin. Oxygenated hemoglobin has two absorption peaks in the visible spectrum, centered on 542 and 577 nm, while deoxygenated hemoglobin has only one absorption peak centered on 556 nm. The total optical absorption is used to reflect the total tissue hemoglobin content. Hence, by scaling the measured absorption spectrum between the known absorption spectra of oxygenated and deoxygenated hemoglobin, the hemoglobin oxygen saturation can be determined. In addition to the sublingual microcirculation, reflectance spectrophotometry is additionally used to clinically measure myocardial oxygenation [36], tissue ischemia [37], or gastric mucosal oxygenation [27].

Microcirculatory parameters

The measurement and quantification of sublingual microcirculatory function can be divided into perfusion or oxygenation parameters. The combination of perfusion and oxygenation measurements provides an integrative overview of microcirculatory behavior, but requires the combined use of different microcirculatory monitoring techniques.

Microcirculatory perfusion parameters

The perfused vessel density (PVD) is an indicator for the proportion of perfused vessels in the microcirculation in relation to the total vessel density (TVD) [38]. The proportion of perfused vessels (PPV) is obtained by dividing PVD by TVD values. The microcirculatory flow index (MFI) is as semi-quantitative determinant of the predominant flow pattern in a certain area of the microcirculation. The MFI ranges from no flow (0), sluggish flow (1), intermittent flow (2) and continuous flow (3) [39]. Figure 2 shows two sidestream dark field images of the sublingual microcirculation of a patient undergoing coronary artery bypass graft surgery before (panel A) and during (panel B) cardiopulmonary bypass. The perfused capillary density has clearly been reduced after cardiopulmonary bypass as indicated by the empty microvessels.

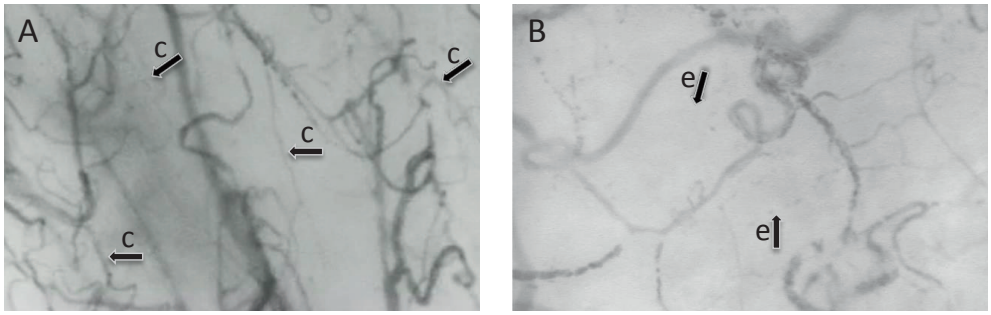

Figure 2 Microcirculatory perfusion in a patient undergoing coronary artery bypass graft surgery with cardiopulmonary bypass. Panel A: before cardiopulmonary bypass; panel B: after cardiopulmonary bypass. There is a clear reduction in perfused capillary density in panel B when compared to the baseline situation. c = capillaries; e = empty capillaries.

A second characteristic of impaired microcirculatory perfusion is an increased heterogeneity of perfusion, both between and within tissues (Figure 3).

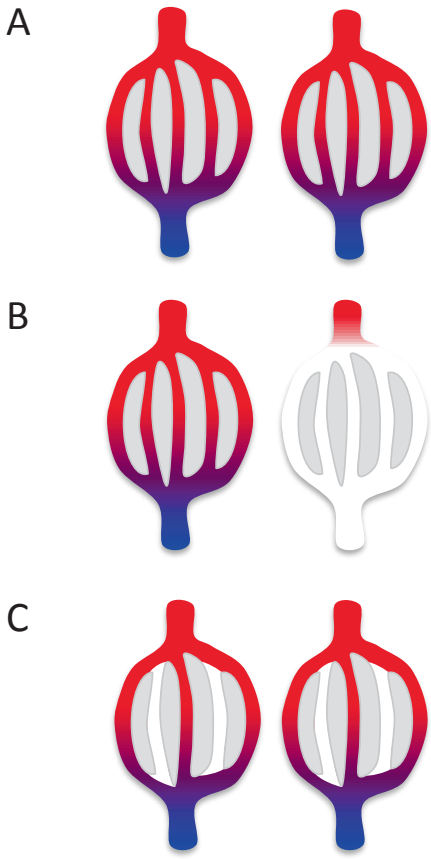

Figure 3 Microvascular perfusion heterogeneity profiles. Panel A shows a normal situation in two separate microcirculation networks. Panel B depicts perfusion heterogeneity between two separate microvascular beds, while panel C illustrates local vascular perfusion heterogeneity within two separate microcirculation networks. Red = oxygenated blood; blue = non-oxygenated blood; grey = nonperfused vessels.

The heterogeneity index can be calculated from the variation among MFI-scores. Trzeciak et al. earlier showed that increased microcirculatory heterogeneity is associated with increased mortality during septic shock [40]. Due to the advances microvascular perfusion imaging the phenomenon of microcirculatory heterogeneity during critical illness is increasingly recognized. However, only few empirical data are currently available, while there are no studies available that assess microcirculatory heterogeneity during cardiac surgery.

Finally, space-time diagrams provide red blood cell velocities in the microcirculation by calculation of the diagram-slopes [41]. The introduction of automated evaluation systems for the assessment of microvascular density and perfusion will finally contribute to objective and uniform quantification of microvascular perfusion abnormalities in the clinical setting [42].

Microcirculatory oxygenation parameters

Near-infrared spectroscopy provides a cerebral tissue oxygenation index (TOI), which is calculated by the formula TOI = O_2Hb/Hb (oxygenated hemoglobin (O_2Hb) divided by the total hemoglobin concentration). A high TOI indicates sufficient oxygen in the tissue, and a low TOI the opposite. The μHbO_2 is a functional indicator of oxygen delivery to the microcirculation and cells. A high μHbO_2 is associated with oxygen off-loading insufficiency by the erythrocytes due to an oxygen diffusion limitation, which may be caused by a diminishment of the number of perfused microvessels. A low μHbO_2 is associated with oxygen delivery insufficiency due to oxygen convection limitation caused by flow disturbances (MFI 0, 1 or 2) in the microvessels [12,14].

Assessment of glycocalyx integrity

The glycocalyx consists of a thin layer of glycoproteins and glycolipids on the plasma membrane of the microvascular endothelium [43,44]. It has been suggested that glycocalyx disruption and damage is associated with the development of cardiovascular disease, but the number of clinical studies on this subject is limited due to the lack of measurement devices. Recently, Vlahu and Vink et al. described a new method to assess endothelial glycocalyx thickness with the use of SDF-imaging in dialysis patients [45]. Using sublingual microcirculatory video recordings, glycocalyx dimensions are estimated by calculation of the perfused boundary region (PBR) in multiple microcirculatory vessels, which is the inverse of the endothelial glycocalyx thickness. Shedding of glycocalyx molecules may lead to an increased perfused boundary region, and is associated with potentiation of inflammatory response and procoagulant conditions.

Specific hemodynamic and metabolic alterations during surgery with cardiopulmonary bypass

The switch from physiological, systemic perfusion to extracorporeal circulation using a heart-lung machine is associated with a sudden change in the nature of the circulatory profile. Among others, these changes include hypotension, hemodilution, hypothermia, hyperoxemia, cardiac arrest and a change from pulsatile to nonpulsatile blood flow. The hemodynamic and metabolic effects associated with extracorporeal circulation may lead to reduced oxygen delivery to vital tissues, functional shunting of the microcirculatory circulation as reflected by the fall-out of red blood cell-carrying capillaries, and enhanced venular flow. These observations suggest diffusional limitation of oxygen transport pathways to the organ tissue. Here we describe the effect of these hemodynamic and metabolic alterations on microcirculatory perfusion and oxygenation.

Blood pressure and cardiac output

A reduction in blood volume due to the transition to extracorporeal circulation, in combination with the systemic inflammatory response, is associated with a decrease in blood pressure and cardiac output, and may lead to hypotensive episodes. Pharmacological correction of hypotension during extracorporeal circulation is often based on the administration of a strong short-acting alpha–adrenergic vasoconstrictor, like phenylephrine. Volume therapy interventions to improve cardiac output include Trendelenburg positioning, passive leg raising or a fluid challenge using crystalloids or colloids.

There is ongoing debate whether hypovolemia and/or systemic hypotension affects microcirculatory perfusion. In healthy volunteers, controlled hypovolemia decreased the perfused vessel density and microcirculatory flow index, which was paralleled by reduced tissue oxygen carrying capacity and oxygenation as measured by NIRS [46]. In contrast, despite a ketanserin-induced vascular resistance reduction and concomitant lowered blood pressure, Elbers et al. showed no effect on the perfused capillary density normovolemic cardiosurgical patients [47]. This study however did not report whether ketanserin also induced an alteration in cardiac output.

In a review by De Backer et al., the relation between system hemodynamics and microcirculatory perfusion was described as relatively loose, especially within the physiological range of cardiac output and blood pressure [10]. However, in case of severe blood pressure derangements, microcirculatory perfusion is additionally affected as was recently shown by two reported cases of nitroglycerin-induced hypotension [48]. Nitroglycerin-induced vasodilation during cardiac surgery was associated with an initial increase in the arteriolar diameter and microcirculatory flow, and followed by a reduction in the microvascular blood velocity during the hypotensive phase [48].

Both volume and pharmacological interventions to correct cardiac output and hypotensive episodes may affect microcirculatory perfusion. Passive leg raising, which induces a fluid shift and increases systemic blood pressure and cardiac output, improved sublingual microcirculatory perfusion in preload responsive severe septic patients [49]. The effects of volume therapy on microvascular recruitment are however difficult to unravel, as they include a blood pressure-modulating and hemodilution component [49]. It has further been suggested that a vasopressor-induced increase in blood pressure in patients with septic shock may even be unbeneficial for microcirculatory perfusion in case of a mean arterial pressure exceeding 65 mmHg, while it will significantly alter cardiac output [50,51]. Indeed, a 20 mmHg increase in systemic blood pressure by phenylephrine was associated with depressed sublingual small vessel blood flow while medium-sized vessels were unaffected [52]. However, from this study it was unclear whether this increase in blood pressure was also associated with augmentation of cardiac output. Others showed an increase in local gastric mucosal blood flow after dopamine infusion, whereas systemic flow remained stable [53].
Intra-aortic balloon pump (IABP) is used in cardiogenic shock to improve mean arterial blood pressure and cardiac output. Although it is assumed that support by IABP counterpulsation improves microcirculatory perfusion, the available studies that focused on the effects of IABP on the microvasculature are conflicting. It has been shown that initiation of IABP therapy is associated with improved microvascular flow [54]. While Den Uil et al. found that temporary discontinuation of IABP did not impair sublingual microcirculatory perfusion or affect red blood cell velocity, discontinuation of IABP support induces an increase in microcirculatory flow in the study of Munsterman et al. [55,56]. However, the latter study focused on patients with a restored body circulation, which suggests that IABP support is only beneficial in patients with impaired systemic hemodynamics.
The aforementioned observations suggest that, in case of a relatively normal systemic blood pressure, volume challenges and vasoactive substances are relatively ineffective as modulators of microcirculatory perfusion. It should however be noted that most investigations did not report whether the applied interventions resulted in alterations in cardiac output, as this requires specialized measurements. Further studies are warranted to reveal new therapeutic strategies for the recruitment of microcirculatory perfusion and oxygenation during hemodynamic derangements.

Hemodilution

Extracorporeal circulation is associated with hemodilution due to the mixture of circulating blood with 1.5-2.0 liters of pump priming solution that result in a reduction in hematocrit values from 38-45% before surgery to 20-28% during cardiopulmonary bypass. The reduction in hematocrit due to the administration of crystalloid or colloid solutions is additionally associated with decreased blood viscosity. Since visualization of capillary perfusion by SDF

imaging is mainly based on the passage of red blood cells through microvessels, the perfused vessel density is consequently altered in case of a lower blood viscosity. Moreover, since pressure-driven microcirculatory perfusion is lowered during a reduction of longitudinal resistance associated with low blood viscosity, red blood cells have difficulty entering high resistance vessels. Indeed, we recently showed that red blood cell transfusion after cardiac surgery, as compared with gelatin-based volume expansion or non-resuscitated patients, increased medium-sized vascular density, red blood cell content and oxygenation in the microcirculation, while the flow index remained unchanged [57]. Moreover, although allogeneic red blood cell transfusion should be reduced to a minimum during cardiac surgery, Yuruk et al. showed that red blood cell administration recruits the microcirculation, leading to improved perfused vessel density and tissue oxygenation [26].

The importance of blood viscosity in maintaining functional capillary density has earlier been shown in experimental studies using hamsters by Cabrales and Tsai, in which they increased blood viscosity while maintaining low hematocrit by adding highly viscous colloids [58,59]. They further showed that the deleterious effects of a reduced functional capillary density due to hemodilution could be reversed by an increase in blood viscosity [58,59]. The beneficial effects of increased blood viscosity are especially attributed to an improvement in shear stress, nitric oxide production and vasoreactivity [60]. In particular, although hemodilution may be expected to be of no consequence for microcirculatory perfusion due to the compensatory increase in cardiac output, extreme hemodilution is however pathophysiological due to the inability of the cardiovascular system to transmit sufficient central pressure to the microcirculation for the maintenance of functional capillary density, which is a linear function of capillary pressure [60]. Although the association of a reduction in hematocrit with adverse outcome and organ dysfunction is well recognized, our insight in the underlying pathophysiological mechanisms are limited, with the presence of an oxygen debt has been suggested as the main cause. The presence of the oxygen debt as a result of increased diffusional distance from filled capillaries to the cellular system instead of a reduced oxygen carrying capacity of blood during low hematocrit states should therefore be further investigated.

Hypothermia

Hypothermia during extracorporeal circulation reduces myocardial and cerebral oxygen consumption, thereby preserving cellular function. In particular, during mild hypothermic extracorporeal circulation the body temperature is decreased to 32-35° Celsius within 15 minutes after the onset of cardiopulmonary bypass. Hypothermic tissue with a temporarily reduced oxygen demand may affect microcirculatory perfusion, and consequently lead to a redistribution of microcirculatory flow to regulate the amount of necessary oxygen in the terminal microcirculatory network. The number of clinical studies focusing on the effects

of hypothermia on microcirculatory perfusion and oxygenation are however limited, and mostly focused on deep hypothermic arrest. A recent experimental study in sheep under normovolemic conditions showed that a 6-hour period of mild hypothermia (34°C) was associated with a reduction of ventricular function, oxygen extraction and microvascular flow when compared to normothermia, which suggested that mild hypothermia may impair tissue oxygen delivery through inappropriate distribution of capillary flow [61]. Finally, microcirculatory parameters recovered in parallel with the correction of temperature [61]. These experimental data suggest that hypothermia partially contributes to an imbalance between oxygen delivery and demand, but clinical studies should further confirm this finding.

Hyperoxemia

Hyperoxemia (20-30 kPa) is applied to compensate for pulmonary bypass and to enhance oxygen delivery to tissues during extracorporeal circulation. However, several lines of investigation have shown that hyperoxemia may have deleterious effects, including a decrease in microvascular functional capillary density [62]. In particular, hamster experiments showed a decrease in functional capillary density under conditions of hyperoxemia, assuming vasoconstriction or shunting proximal to the capillary network [63,64]. Tsai et al. explained this phenomenon by demonstrating vasoconstriction of arterioles without a concomitant reduction in oxygen delivery in the microcirculation, which would suggest a compensatory mechanism that is regulated upstream of the capillary level [64]. There are currently no clinical studies that evaluated the effects of hyperoxemia on the human microcirculation using SDF imaging or reflectance spectrophotometry.

Cardiac arrest

Cardiopulmonary arrest to enable coronary artery bypass grafting is considered as a period of myocardial ischemia, which may affect the microvasculature. There is however no literature available with respect to microcirculatory alterations during intraoperative cardioplegia-induced arrest. In situations of complete circulatory arrest it has been shown that sublingual microcirculatory perfusion significantly reduces, and continuation of these microcirculatory perfusion derangements is associated with poor patient outcome [65]. The direct effects of hypothermic circulatory arrest on microcirculatory perfusion were studied in patients undergoing aortic arch reconstruction. Circulatory arrest was associated with an immediate shutdown of sublingual microvessel perfusion, while flow in larger microvessels persisted [66]. This is paralleled by a cerebral decrease in oxygenated hemoglobin, increase in deoxygenated hemoglobin and a reduction in the cerebral oxygen extraction ratio [67]. Impaired microcirculatory perfusion and oxygenation are however recovered after restoration of myocardial function and systemic blood flow.

Nonpulsatile flow conditions

The switch to extracorporeal circulation is associated with a change from pulsatile to nonpulsatile flow conditions. Recently, our group has demonstrated that nonpulsatile CPB is associated with reduced microcirculatory perfusion, which was most notable after discontinuation of extracorporeal circulation [68]. These findings were later confirmed by a study by O'Neil et al. [69]. In contrast, others found that short-term administration of pulsatile flow during cardiopulmonary bypass was not beneficial for microcirculatory perfusion [70] or cerebral oxygenation [71] when compared to a laminar flow conditions. However, the study design of these studies was suboptimal due to administration of a combination of distinct flow modalities to individual patients [72]. Pulsatile flow during cardiopulmonary bypass additionally seemed to be beneficial as reflected by reduced markers of endothelial damage and improved gastric mucosal oxygenation and tonometry [73,74]. Moreover, isolated resistance vessel exposed to nonpulsatile flow demonstrated vascular inflammation and generation of reactive oxygen species within 30 minutes, even in the absence of blood components [75]. These detrimental effects were absent during exposure to pulsatile flow. Finally, in larger studies it was confirmed that pulsatile flow was associated with better clinical outcome than nonpulsatile flow during CPB [76,77].

Proinflammatory conditions

The effects of sepsis and the systemic inflammatory response syndrome on microcirculatory behavior are beyond the scope of this review, and extensively summarized by others [78]. The onset of cardiopulmonary bypass leads to contact activation and the induction of proinflammatory mediators. In addition, extensive surgical trauma during cardiac procedures may further contribute to a proinflammatory state. Although the specific contribution of inflammation to microcirculatory perfusion derangements is currently unrevealed, there are indications that a proinflammatory state may be associated with microcirculatory dysfunction. Vlahu et al. showed that patients with high C-reactive protein levels (> 10 mg/l) had an increased microvascular perfused boundary region as compared to patients with low C-reactive protein levels [45]. It may therefore be conceivable that perioperative interventions aimed at reducing inflammatory activation may be effective for maintaining microcirculatory patency.

The microcirculatory response during cardiac surgery

Cardiac surgery with cardiopulmonary bypass

Cardiopulmonary bypass is associated with hemodynamic and metabolic alterations that may influence microcirculatory perfusion and oxygenation. Although individual hemodynamic and metabolic parameters, such as hypotension, hemodilution and hypothermia, and pharmacological substances like anesthetics [79] may have distinct effects on microcirculatory behavior, most clinical studies focus on the overall accumulation of microcirculatory responses during cardiopulmonary bypass.

We earlier showed that the imposition of extracorporeal circulation during coronary arterial bypass grafting reduced perfused capillary density and increased venular blood velocity [25]. We further observed a rise in microvascular hemoglobin oxygen saturation, possibly due to a defect in oxygen extraction [25]. Others showed that cardiac surgery with cardiopulmonary bypass is associated with a decreased proportion and density of perfused small vessels, and these observations are irrespective of hemodynamic changes [80-82]. In contrast, Maier et al. demonstrated that initiation of cardiopulmonary bypass did not alter sublingual microcirculatory perfusion, while an additional phenylephrine-induced systemic blood pressure increase reduced small vessel blood flow and augmented tissue hemoglobin oxygenation [52]. Others showed that the rectal microvascular flow index and the proportion of perfused vessels was almost normal at 30 minutes following cardiac surgery [23].

Using reflectance spectroscopy during different phases of coronary artery bypass grafting with cardiopulmonary bypass, it was further shown that tissue oxygenation decreases during cardiac arrest, while it is augmented after reperfusion [36]. Moreover, cardiopulmonary bypass is associated with a reduction in palmar tissue oxygenation [83]. Fournell et al. found a sustained decrease in gastric mucosal oxygen saturation following cardiopulmonary bypass using reflectance spectrophotometry [27]. The cardiopulmonary bypass-associated changes in systemic, microvascular and hemorheologic variables are presented in Table 1.

Off-pump coronary artery bypass graft surgery

Off-pump coronary artery bypass graft surgery (OPCAB) is associated with a decrease in microcirculatory perfusion, although to a lesser extent than observed during cardiac procedures with extracorporeal circulation [80]. In addition, cardiac displacement during off-pump surgery induces acute changes in microcirculatory perfusion characteristics. Cardiac displacement during off-pump coronary artery bypass graft surgery for posterior and anterolateral graft anastomoses is associated with a reduction in cardiac output of 15-45%. The reduction in cardiac output may be associated with cessation of microcirculatory blood flow and decreases in microcirculatory hemoglobin oxygenation. Indeed, we earlier showed that off-pump procedures are associated with distinct alterations in microcirculatory

function when compared with cardiac surgery with extracorporeal circulation [24,25]. In particular, cardiac displacement during off-pump surgery did not affect capillary density, but resulted in cessation of microcirculatory flow due to a reduced entry of red blood cells into the microvasculature, and decreased hemoglobin oxygen saturation in parallel to the sudden decrease in cardiac output as a result of cardiac displacement [24,25]. The results in off-pump patients during cardiac positioning show that oxygen availability in the sublingual microcirculation is reduced due to the failure of red blood cells entering the capillaries, while we observed a redistribution of blood with concomitant hemodilution at the onset of cardiopulmonary bypass in cardiac surgery. Moreover, cardiac displacement is responsible for a reduction in cerebral cortical oxygenation that is reversed by returning the heart to its natural position [84]. One may assume that cardiac positions that cause the largest impairment of systemic hemodynamics lead to the most abundant microcirculatory disturbances. The overall effects of cardiac displacement during off-pump cardiac surgery on the microcirculation are shown in Table 2.

Table 2 Effects of on-pump and off-pump cardiac surgery on systemic, microcirculatory and hemorheologic variables.

Description	**Cardiopulmonary bypass** (on-pump surgery)	**Cardiac displacement** (off-pump surgery)
Systemic variables		
Temperature	↓	=
Hemoglobin	↓	=
Cardiac output	↑	↓
Blood pressure	↓	↓
Oxygen delivery	↓	↓
Microcirculatory variables		
Hemoglobin concentration	↓	=
Hemoglobin O_2 saturation	↑	↓
Red blood cell velocity	↑	↓
Perfused capillary density	↓	=
Hemorheologic variables		
Hematocrit	↓	=
Blood viscosity	↓	=

Minimal extracorporeal circulation

Minimal extracorporeal circulation in CABG surgery is associated with reduced hemodilution when compared to conventional cardiac surgery with cardiopulmonary bypass. Although conventional and minimal extracorporeal circulation with aortic cross-clamping are both associated with a decrease in the functional capillary density, a faster recovery of microvascular perfusion was observed in the minimal extracorporeal circulation group when

compared to conventional surgery [85,86]. These findings suggest that the use of a minimized cardiopulmonary bypass system may be of small benefit for the microcirculation in patients undergoing cardiac surgery.

Integrative monitoring of microcirculatory function during cardiac surgery

Despite the available literature, a better understanding of derangements in microcirculatory perfusion and tissue oxygenation is required to avoid microcirculatory dysfunction during cardiac surgery. An integrative evaluation of sublingual microvessel perfusion in combination with microcirculatory oxygenation provides novel insight in the effect of iatrogenic maneuvers during cardiac surgical procedures on microcirculatory perfusion and blood and oxygen supply. Moreover, this integrative perioperative monitoring of the microcirculation may be beneficial to obtain a better definition of microcirculatory dysfunction during acute events like cardiac surgery. The introduction of automated analysis software to quantify microcirculatory alterations [42,45], and the validation of novel, user-friendly measurement devices, like transcutaneous pCO_2 measurements [16], may further contribute to the development of microvascular disturbances as clinical endpoint.

Conclusion

The cardiosurgical setting is an interesting field to gain more insight in microvascular derangements, as surgical procedures are based on well-defined procedures and interventions and the effect of anesthetic and surgical management on microcirculatory function is predictable. Moreover, perioperative systemic hemodynamic alterations are acute and causative for microvascular effects, and subsequent corrections of these alterations are reflected by the microcirculation. This review shows that we gained more insight in the acute effects of cardiac surgery with or without cardiopulmonary bypass on microcirculatory perfusion and oxygenation over the last decade.

The microcirculation is particularly compromised during and after extracorporeal circulation. The microvasculature is less affected during off-pump cardiac surgery when compared to procedures with the use of cardiopulmonary bypass. Changes in cardiac output during cardiac displacement may however induce an acute, transient reduction in microcirculatory perfusion during off-pump surgery. Inflammatory and endothelial activation is thought to be the major determinant of impaired microcirculatory perfusion during cardiopulmonary bypass. The available literature suggests that infusion of vasoactive agents hardly seems to influence microcirculatory alterations during cardiac surgery. Moreover, restrictive perioperative fluid administration is advised, as higher hematocrit levels are associated with both improved microcirculatory perfusion and oxygenation. Moreover, pulsatile flow during cardiopulmonary bypass leads to improved microvascular recovery, and is additionally associated with favorable patient outcome. Our knowledge with respect to the role of microvascular perfusion heterogeneity in the development of tissue hypoperfusion and hypo-oxygenation is however still limited. Moreover, the lack of large patient studies with a follow-up period of one or more consecutive post-surgical days prohibits a robust conclusion with respect to the consequences of microcirculatory responses during cardiac surgery with or without cardiopulmonary bypass on outcome.

Future clinical investigations of interventions that preserve microcirculatory perfusion in the intraoperative and postoperative period are warranted to gain more insight in the impact of microvascular tissue hypoperfusion on patient recovery. This may additionally enhance our knowledge regarding the predictive value of early microcirculatory perfusion disturbances for the development of postoperative complications in patients undergoing cardiac surgery.

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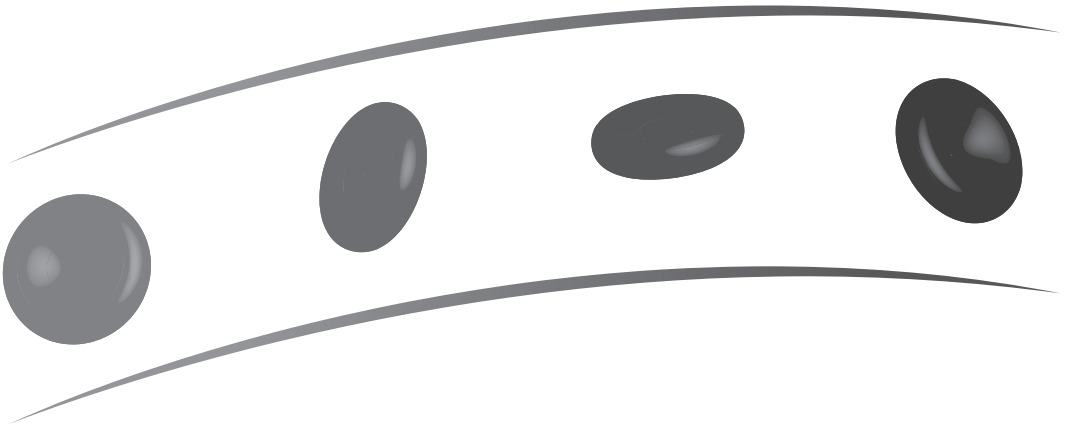

CHAPTER 3

Microcirculatory perfusion is preserved during off-pump but not on-pump cardiac surgery

N.J. Koning, A.B.A. Vonk, M.I. Meesters, T. Oomens, M. Verkaik, E.K. Jansen, C. Baufreton, C. Boer.

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Abstract

Background: This study investigated the perioperative course of microcirculatory perfusion in off-pump as compared to on-pump surgery. Additionally, the impact of changes in systemic hemodynamics, hematocrit and body temperature was studied.

Methods: In this prospective, non-randomized, observational study we included patients undergoing coronary artery bypass grafting with (n=13) or without (n=13) use of cardiopulmonary bypass. Microcirculatory measurements were obtained with sublingual Sidestream Darkfield (SDF) imaging at five time points ranging from induction of anesthesia to intensive care unit (ICU) admission.

Results: Despite a comparable reduction in intraoperative blood pressure among groups, the perfused vessel density decreased more than 20% after onset of extracorporeal circulation while it remained stable in the off-pump group. The reduction in microvascular perfusion in the on-pump group was further paralleled by a decreased hematocrit and temperature. Although post-bypass hematocrit levels and body temperature restored to similar levels as in the off-pump group, the median microvascular flow index remained reduced after bypass (2.4 (2.3-2.7)) compared to baseline (2.8 (2.7-2.9); P=0.021).

Conclusion: Microcirculatory perfusion remained unaltered throughout off-pump surgery. In contrast, microvascular perfusion declined following initiation of cardiopulmonary bypass and did not recover in the early postoperative phase.

Introduction

Cardiopulmonary bypass during cardiac surgery is associated with disturbances in microcirculatory perfusion and oxygenation [1-4]. In addition to the primary microcirculatory injury related to contact activation by extracorporeal circulation and absence of pulsatile flow, on-pump procedures result in hemodilution, which independently deteriorate microvascular function [2,5,6,7,8].

In contrast, off-pump coronary surgery is associated with preserved hematocrit levels and pulsatile flow generated by the beating heart, contributing to preservation and maintenance of microcirculatory perfusion [9]. Moreover, extracorporeal contact activation is absent in off-pump surgery. On the other hand, cardiac positioning during off-pump procedures is associated with short-lasting cessation of microcirculatory flow [3]. Whether multiple positioning events during off-pump surgery lead to more enduring disturbances in microvascular perfusion is still unknown.

Current literature is limited regarding microcirculatory perfusion dissimilarities between on-pump and off-pump procedures, in particular with respect to the course throughout the intraoperative period [1,3,10]. We previously showed that patients undergoing on-pump coronary surgery develop a decrease in microcirculatory perfusion following the onset of cardiopulmonary bypass, irrespective of pulsatile or nonpulsatile perfusion [4]. Although De Backer et al. suggested that microcirculatory alterations are most pronounced during surgery with cardiopulmonary bypass, they have not performed intraoperative measurements in off-pump patients, prohibiting a close comparison between groups [1]. In the present study we therefore compared intra- and early postoperative microcirculatory perfusion in patients undergoing on-pump and off-pump coronary artery bypass graft (CABG) surgery. Additionally, we examined the relationship between microcirculatory alterations during both surgical modalities and intraoperative hematocrit or body temperature. We hypothesized that use of cardiopulmonary bypass during on-pump surgery induces more severe and enduring disturbances of microcirculatory perfusion as compared to off-pump surgery, which are not fully explained by systemic hemodynamics, hematocrit and body temperature.

Methods

Patient characteristics

This single center, non-randomized clinical study was approved by the local Human Subjects Committee of our university medical center, and written informed consent was obtained from all participants. The two study groups consisted of patients undergoing coronary artery bypass graft (CABG) surgery with nonpulsatile cardiopulmonary bypass (CPB; n=13) or off-pump beating heart CABG procedures (OPCAB; n=13) in the period March 2010 to March 2012. Exclusion criteria were previous heart surgery, emergency surgery, insulin-dependent diabetes mellitus and a body mass index over 30 kg/m^2. Assignment to OPCAB procedures was based on the operating schedule. OPCAB surgery was performed by a single surgeon specialized in this type of procedure (EKJ). Surgery with CPB was randomly performed by one of the four other surgeons in our cardiosurgical department.

Anesthesia protocol

The anesthesia protocol for CPB and OPCAB procedures was similar and earlier described by our group [4]. Briefly, platelet inhibitors were continued until five (clopidogrel) and one (acetylsalicylic acid) days preceding surgery. Following lorazepam administration (5 mg), anesthesia was induced using sufentanil (3-7 µg/kg), pancuronium bromide (0.1 mg/kg) and midazolam (0.1 mg/kg) and maintained by continuous propofol infusion (200-400 mg/h). Ventilation parameters consisted of 8-10 ml/kg tidal volume, 4-5% end-tidal CO_2, 45% inspiratory O_2 and positive end-expiratory pressure of 5 cm H_2O. After anesthesia induction, patients received dexamethasone (1 mg/kg) and cefazolin (1 g). In patients undergoing CPB surgery, cardiac output was monitored intermittently using a thermodilution pulmonary artery catheter. For OPCAB surgery, all patients received a pulmonary artery catheter for continuous cardiac output monitoring (Vigilance monitor, Edwards Lifesciences, USA). In all patients, a cell saving device was used for retransfusion of pericardial shed blood. At the end of surgery, heparin was reversed with protamine and two grams of tranexamic acid were administered as antifibrinolytic therapy.

Cardiopulmonary bypass

Cardiopulmonary bypass (CPB) was performed with a S5 heart-lung machine with heater-cooler device (Stöckert Instrumente GMBH, Munich, Germany) and centrifugal pump (Sarns, Terumo Europe NV, Leuven, Belgium), combined with a heparin coated polyvinyl tubing system with a hollow fiber oxygenator and arterial line filter (Affinity, Medtronic, Minneapolis, MN, USA). Priming of the circuit was performed with 1000 ml modified fluid gelatin (Braun Melsungen AG, Germany), 250 ml lactated Ringer's solution (Baxter BV, Utrecht, Netherlands), 100 ml mannitol (20%, Baxter BV) and 50 ml sodium bicarbonate (8.4% Braun Melsungen AG), 1000

mg cefalozin (Eli Lilly Nederland BV, Nieuwegein, Netherlands) and 5000 IU porcine heparin. Nonpulsatile CPB (34°C; 2.2-3.0 l/min/m^2) started following heparin administration (300 IU/kg) when the activated clotting time (ACT) exceeded 480 s. Cardiac arrest was induced by 4°C crystalloid cardioplegia solution (St. Thomas, VU University Medical Center, Amsterdam, the Netherlands). Patients were weaned from CPB when rectal temperature was above 36°C.

Off-pump surgery

In OPCAB surgery, an initial heparin dose of 300 IU/kg was administered. During grafting, the ACT was maintained above 380 s. Patients were kept normothermic throughout surgery. OPCAB-procedures were performed with two deep pericardial stitches and a mechanical stabilizer (Ultima, Maquet, Hilversum, The Netherlands). To avoid systemic hypoperfusion, cardiac repositioning was performed when systolic blood pressure dropped under 70 mmHg or when venous oxygen saturation declined under 60% [11].

SDF-imaging

Sublingual mucosal microcirculation measurements were performed during surgery and at the intensive care unit (ICU) using Side Dark Field (SDF) imaging (MicroScan, Microvision Medical, Amsterdam, Netherlands), as previously described [3,4]. During CPB surgery, images were made after induction of anesthesia (T1), 10 minutes after cross-clamping the aorta during cardiopulmonary bypass (T2), 10 minutes before removing the aortic cross-clamp during cardiopulmonary bypass (T3), after weaning from CPB, at the operation theater (T4) and in the first hour on ICU, while on mechanical ventilation (T5). In the OPCAB surgery group, measurements were performed after induction of anesthesia (T1), during the grafting of the first and second anastomoses (T2 and T3, respectively), at the end of surgery (T4) and in the first hour on ICU, while on mechanical ventilation (T5). In order to be able to perform comparisons of changes in perioperative microcirculatory perfusion, measurement time points for both CPB and OPCAB groups were named according to CPB surgical events. At each time point, 3 sequences of 10 seconds were recorded.

Analysis was performed blinded for study group as described earlier [4]. In brief, automatic vascular analysis software (AVA 3.0, Microvision Medical, Amsterdam) was used to determine parameters of relevance, according to general consensus [12]. All vessels were identified manually for total vessel density (TVD). Subsequently each vessel was individually scored for its flow character to obtain perfused vessel density (PVD). Finally, videos were analyzed for Microvascular Flow Index (MFI). This is a semi-quantitative score, in which the flow score (no flow – sluggish flow – intermittent flow – continuous flow) that is demonstrated by the majority of small vessels is assigned to a quadrant of the video screen. Scores of the four quadrants were averaged per video. All scores were calculated for small vessels (diameter < 20 µm) to enable specific focus on the microvasculature important for oxygen exchange.

Collection of systemic parameters and additional data

From all patients, intra- and early postoperative hematocrit levels, administered vasoactive medication and systemic variables, including temperature, mean arterial pressure and cardiac index, were recorded. Moreover, lactate levels in arterial blood gases were determined at baseline (T1) and at the end of surgery (T4).

Statistical analysis

The study sample size was based on previous findings of our group, showing a 23% reduction in perfused small vessel density during cardiopulmonary bypass with a standard deviation of up to 15% [3]. A power of 90% and an alpha of 0.01 were used in the sample size calculation. The primary outcome parameter was sublingual perfused small vessel density. Data were analyzed using SPSS statistical software package (17.0; IBM, New York, USA). All values are expressed as mean ± standard deviation or median with interquartile range (IQR). Repeated-measures (RM) ANOVA was performed to analyze time-dependent differences between groups. Differences between groups at individual time points were analyzed by Student's T-tests for parametric data or Mann-Whitney U tests for nonparametric data. Within-group differences were tested with paired T-tests or Wilcoxon test for parametric and nonparametric data, respectively. Correlations between changes in hemodynamic variables, hematocrit or temperature and alterations in microcirculatory variables during onset of CPB or weaning from CPB were tested with Pearson correlations tests. $P < 0.05$ was considered as statistically significant.

Results

General patient characteristics

Group characteristics are presented in Table 1. Overall, patient demographics were similar among groups, however patients undergoing OPCAB surgery received less heparin and protamine throughout the procedure as compared to CPB surgical patients.

Table 1 Patient characteristics

Description	Nonpulsatile CPB	Off-pump	P-value
N	13	13	
Age (years [median (IQR)])	65 (62-72)	63 (54-73)	0.411
Males [n] (%)	12 / 13 (92%)	13 / 13 (100%)	1.000
BSA (m^2)	2.0 ± 0.2	2.0 ± 0.2	0.896
Diabetes Mellitus II [n] (%)	2 / 11 (15%)	1 / 13 (8%)	1.000
Euroscore [median (IQR)]	3 (2-5)	3 (1-5)	0.900
Surgery time (min)	248 ± 38	250 ± 24	0.890
CPB time (min)	106 ± 24	NA	NA
Cross-clamp time (min)	67 ± 15	NA	NA
Anastomoses (n)	4 (3-5)	4 (3-5)	1.000
Total heparin dose (IU/kg)	502 (425-582)	286 (267-358)	0.000*
Protamine dose (mg)	430 (369-572)	250 (222-355)	0.001*
Blood products (RBC / FFP / PLT; [n])	16 / 8 / 5	11 / 6 / 3	n.s.

Values represent mean ± SD, median with interquartile range or frequencies. Numeric data were tested with Students T-test between groups. Nonparametric data were tested with a Mann-Whitney U test, whereas nominal data were tested with a Chi-square test. BSA = body surface area, CPB = cardiopulmonary bypass, IQR = interquartile range, N = number, NA = Not applicable.

Hemodynamic and perioperative parameters

Perioperative values of hematocrit (panel A) and temperature (panel B) are shown in figure 1 for CPB and OPCAB groups. Cardiopulmonary bypass was associated with an intraoperative reduction in temperature, whereas no alterations were observed during OPCAB surgery (ANOVA RM: P=0.001). Hematocrit declined following onset of extracorporeal circulation as compared to baseline (P<0.001) and was lower than in the OPCAB group after weaning from cardiopulmonary bypass (0.28 ± 0.04 l/l vs. 0.38 ± 0.05 l/l, respectively; P<0.001). At ICU, hematocrit levels in the CPB group restored towards hematocrit levels as observed in the OPCAB group (0.33 ± 0.03 l/l vs. 0.35 ± 0.04 l/l, respectively; P=0.223). The courses of cardiac index (panel C) and mean arterial pressure (panel D) did not reveal differences between groups over the course of the study.

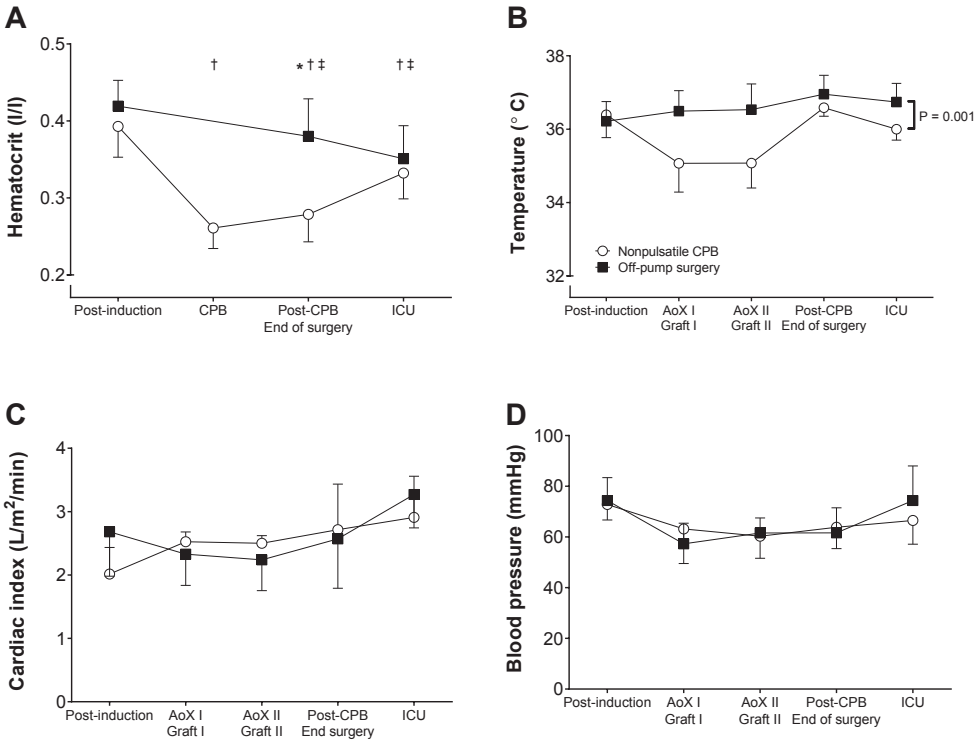

Figure 1 Perioperative course for hematocrit (panel A), temperature (panel B), cardiac index (panel C) and mean arterial pressure (panel D). The X-axes contain labels for both on-pump (above) and corresponding OPCAB (below) events. Following onset of extracorporeal circulation, the nonpulsatile CPB group (white circles) was associated with decreased temperature (P=0.011) and hematocrit (P<0.01 for T=4) as compared to the OPCAB group (OP; grey squares). No differences between groups were observed for cardiac index or mean arterial pressure. Data represented in panel A were evaluated using Student's T-tests and paired T-tests. Data in panels B, C and D were analyzed by repeated measures ANOVA. * P < 0.05 CPB vs OPCAB, † P < 0.05 CPB vs T1; ‡ P < 0.05 OPCAB vs T1.

Dopamine or nitroglycerin infusion was not different between groups (Table 2).

Table 2 Vasoactive medication

		Post-induction	AoX I	AoX II	Post-CPB	ICU
		T1	T2	T3	T4	T5
CPB	**NTG**	0.6 ± 0.6	0.1 ± 0.1	0.3 ± 0.3	0.8 ± 0.7	0.0 ± 0.0
	Dopamine	1.0 ± 0.9	0.3 ± 0.6	0.6 ± 0.9	1.5 ± 1.2	3.1 ± 1.7
OPCAB	**NTG**	0.3 ± 0.4	0.5 ± 0.9	0.3 ± 0.4	0.4 ± 0.4	0.0 ± 0.0
	Dopamine	0.9 ± 1.3	1.3 ± 1.4	1.5 ± 1.8	1.4 ± 1.6	1.3 ± 2.4

Infusion rates of NTG (0.5 mg/ml) and dopamine (4 mg/ml) in ml/hour. Values are mean ± SD. Data were tested with a Mann-Whitney U test. AoX = Aortic cross-clamp, CPB = cardiopulmonary bypass, ICU = Intensive Care Unit, NTG = nitroglycerin, OPCAB = Off-pump coronary artery bypass.

Microcirculatory perfusion

Figure 2 represents the median microvascular flow index and total vessel density and perfused vessel density of the sublingual mucosal microvasculature with a diameter < 20 µm.

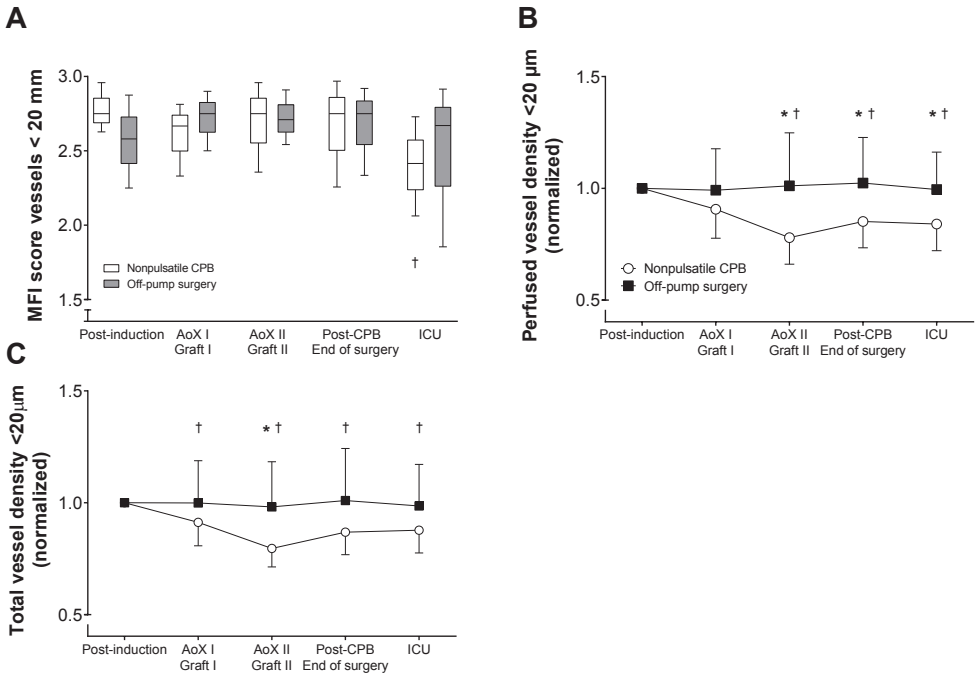

Figure 2 Microcirculatory perfusion parameters represented by microcirculatory flow index (panel A), total (panel B) and perfused (panel C) vessel density. The x-axis contains labels for both CPB (above) and corresponding OPCAB (below) events. The microvascular flow index showed no differences between groups. Total and perfused vessel density declined in the CPB group (CPB; white circles) after initiation of extracorporeal circulation, no recovery was observed following weaning from cardiopulmonary bypass. Microcirculatory perfusion in the OPCAB group (OP; grey squares) showed no alterations throughout the study period. The microvascular flow index was tested with Mann-Whitney U tests for all individual time points and Wilcoxon tests for within group comparison. Data in panel B and C were tested with Student's T-tests and paired T-tests. * $P < 0.05$ CPB vs OPCAB; † $P < 0.05$ CPB vs T1.

The microvascular flow index (Panel A) remained unaltered throughout the study period in the OPCAB group. The MFI score tended to reduce after initiation of extracorporeal circulation from 2.8 (2.7-2.9) to 2.7 (2.4-2.7, P=0.241). Upon intensive care unit admission, the MFI-score in CPB patients was decreased as compared to baseline levels (2.4 (2.3-2.7), P=0.021 versus baseline).

Most notable, total (panel B) and perfused (panel C) small vessel density during OPCAB surgery showed no intraoperative or postoperative reductions as compared to baseline. Total and perfused vessel density decreased by 8% (P=0.044 and P=0.085, respectively) after onset of extracorporeal circulation in the CPB group (T=2). Following a period of nonpulsatile flow during cross-clamp time, perfused vessels declined to 78 ± 12 % of baseline level (P=0.004), whereas perfused vessel density was not reduced at the corresponding time point during OPCAB surgery (101 ± 24 %; P=0.005 between groups). The postoperative perfused vessel density was higher in the OPCAB group than in the CPB group at T=4 (post-CPB; 102 ± 20 % vs. 85 ± 12 % respectively; P=0.018) and T=5 (ICU; 99 ± 17 % vs. 84 ± 12 %, respectively; P=0.015; ANOVA RM: P=0.015). In the nonpulsatile CPB group, no restoration of perfused capillary density after extracorporeal circulation was observed, although hematocrit levels approached the hematocrit values of the OPCAB group at this time point. Lactate concentrations increased from 0.88 ± 0.31 mmol/l at baseline to 1.38 ± 0.49 mmol/l at the end of CPB surgery (T1 vs T4; P=0.032), whereas lactate levels were not augmented after OPCAB surgery (T1: 1.08 ± 0.36 mmol/l versus T4: 1.25 ± 0.42 mmol/l, n.s.)

Correlations

In the OPCAB group, no correlations could be examined due to stable course of both microvascular perfusion and other perioperative variables. In the CPB group, no correlations were found between changes in mean arterial pressure, cardiac index, temperature or hematocrit and alterations in microcirculatory perfused vessel density of flow index during onset of CPB or weaning from CPB (not significant for all correlations).

Discussion

The present study shows that OPCAB surgery was associated with preserved perioperative microcirculatory perfusion parameters. In contrast, onset of cardiopulmonary bypass is associated with a deteriorating effect on microcirculatory perfusion, most notable at the end of aortic cross-clamp time. This observation continued throughout the early postoperative phase, and was not reversed by normalization of hematocrit or intraoperative body temperature. Decreased microvascular perfusion in patients undergoing nonpulsatile cardiopulmonary bypass was predominantly reflected by a decrease in perfused microvascular density, additionally semi-quantitative flow scores were reduced upon ICU admission. A reduction in perfused vessel density with relatively preserved microcirculatory flow index scores in the CPB group after onset of extracorporeal circulation may suggest a cause within the microcirculation rather than a driving macrocirculatory origin. We found a perioperative increase in lactate levels in the group undergoing cardiopulmonary bypass, but not in the OPCAB group.

Mean arterial pressure and cardiac index showed a similar course in both groups and yielded no correlation with microcirculatory parameters. This is in line with abundant evidence showing the independence of the microcirculation from the macrocirculation within the observed pressure range [1,3,8]. As previously mentioned, it seems that the macrocirculatory driving force within the normal ranges as observed in cardiac surgery plays a minor role in the regulation of microvascular density and flow.

Bauer et al. previously suggested that hemodilution may directly result in disturbances of microcirculatory perfusion during cardiopulmonary bypass [2]. Their investigations were however limited by the lack of a control group without cardiopulmonary bypass [2]. In the present study, hemodilution occurred simultaneously with a decrease in microvascular density and flow, whereas both phenomena were absent in the OPCAB group. Consequently, it is conceivable that an acute hematocrit reduction contributes to decreases microcirculatory perfusion. This is supported by animal studies, which revealed that isovolemic hemodilution decreases microvascular density [6,13]. In recent human studies comparing conventional cardiopulmonary bypass with miniaturized extracorporeal circulation systems, reduced hemodilution after onset of CPB was associated with improved post-CPB microvascular recovery in a blinded study [14] and unaltered perioperative microcirculatory parameters in an unblinded study [15]. Although acute hemodilution may play a role in the initial decrease of microvascular perfusion, the approximation of hematocrit levels in the present study between patients undergoing nonpulsatile CPB and OPCAB procedures upon intensive care admission were not associated with similar perfused vessel density levels in the present study. Initiation of extracorporeal circulation was paralleled by a sudden decrease in perfused microvascular density that was absent in the OPCAB group. Discontinuation of cardiopulmonary

bypass, however, did not acutely influence microcirculatory perfusion parameters. Activated blood components that leave lasting disturbances of microcirculatory perfusion could be explanatory for the impaired recovery after nonpulsatile cardiopulmonary bypass. This is in line with previous studies, which show that endothelial activation markers and cytokines can be augmented throughout the first 24 hours after weaning from nonpulsatile cardiopulmonary bypass [16,17]. Moreover, endothelial activation parameters did not increase during or after OPCAB surgery, in parallel with the unaltered microcirculatory perfusion currently described [17].

It is unlikely that mild hypothermia contributes to decreased microcirculatory perfusion during CPB. Indeed, consistent with the current study, others found no correlation between perfused vessels and temperature or hematocrit in patients undergoing cardiopulmonary bypass [1]. As the initiation of cardiopulmonary bypass is accompanied by reduced body temperature, it is expected that cellular metabolism and oxygen demand decrease [18], with concomitant decreased microcirculatory blood flow. In contrast, we observed obstructed capillaries, which may not be expected during hypothermia. Moreover, an experimental study showed that long-lasting mild hypothermia can lead to transient microvascular disturbances, which restored in parallel with correction of temperature [19]. As immediate microvascular recovery did not take place in nonpulsatile CPB surgery, we believe temperature within the observed range plays a negligible role in relation to microcirculatory perfusion.

Previously, our group reported on microcirculatory disturbances during short-lasting cardiac displacement in OPCAB surgery [3,11]. Reductions in mean arterial pressure and cardiac output of 40% to 50% were associated with decreased microvascular blood velocity and oxygenation in the sublingual microcirculation [3]. Moreover, cerebral oxygenation temporarily reduced with 17% [11]. However, no information was available regarding follow-up on microcirculatory perfusion after disturbances induced by cardiac displacement during OPCAB surgery. A report of De Backer et al. on microcirculatory perfusion in patients undergoing CPB and OPCAB surgery, concluded that microcirculatory disturbances were present, regardless of whether CPB was used [1]. However, no intraoperative microcirculatory measurements were made in the OPCAB group, leaving a wide gap of information between induction of anesthesia and ICU admission [1]. Intraoperative measurements would have been of vital importance to support their conclusion that there is no difference in microcirculatory perfusion between study groups [1]. The present study is the first to show the intraoperative course of microvascular perfusion during OPCAB surgery. Despite a similar amount of surgical trauma that is inflicted in both groups, a clearly favorable pattern of microcirculatory perfusion is observed in the OPCAB group as compared to the CPB group. Unchanged postoperative lactate levels following OPCAB surgery confirm these findings. In line with these results, Loef et al. found that OPCAB-surgery indeed attenuated sensitive markers of kidney injury as compared to CPB-surgery [20], while others showed no difference in creatinine clearance in a large study comparing patients undergoing OPCAB-surgery or CPB-surgery [21].

Cardiopulmonary bypass-induced decrease of microvascular density might also partially be attributed to loss of pulsatility. We earlier showed that extracorporeal circulation with pulsatility is however unable to prevent an initial decrease in perfused vessels, although it contributes to improved recovery of microvascular density and flow during bypass [4].

A weakness of the current study is that patients could not be randomized between CPB and OPCAB surgery, as only one surgeon was specialized in OPCAB-surgery. Therefore allocation took place based on the operating schedule, however there was no selection on patient conditions. However, we do not believe that this hinders our conclusions, as no major preoperative differences existed between groups. Although the study was able to demonstrate the differences in the main outcome parameters on which the power calculation was based, the sample size of our study was small. In order to improve perioperative microcirculation in patients, more insight is needed in microcirculatory perfusion during clinical situations. An interesting subject of further research would be microcirculatory investigation in patients undergoing hypothermic versus normothermic CPB surgery.

Although the exact clinical relevance of the current results are unknown in a patient population with relatively few complication, in recent years it has become obvious that maintenance of microcirculatory perfusion is an important goal in critical care [22,23]. Indeed, no studies relating microcirculatory perfusion and outcome in cardiac surgical populations have been performed. Currently, pulsatile flow during CPB is the only intervention that has proved to be effective on both microcirculatory improvement and on clinical outcome in independent studies [4,24]. We believe striving for further strategies based preservation of physiological microcirculatory circumstances is warranted based on the results in septic patients.

In conclusion, the current study demonstrates that microvascular density and flow remain unaltered throughout OPCAB surgery, whereas nonpulsatile cardiopulmonary bypass is associated with disturbed perioperative microcirculatory perfusion. Microcirculatory disturbances following cardiopulmonary bypass did not resolve when hematocrit levels and mild hypothermia were corrected. From a microcirculatory perspective, OPCAB surgery yields less perfusion disturbances than cardiac surgery with CPB.

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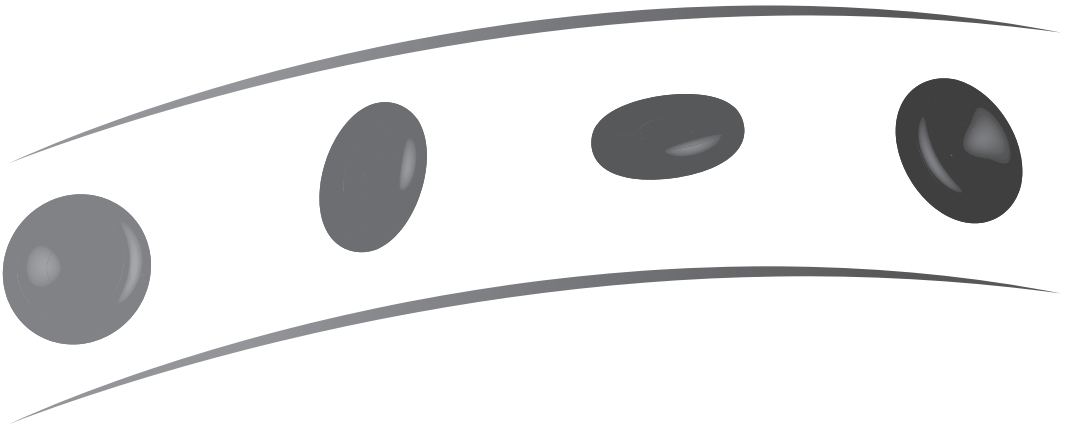

CHAPTER 4

Pulsatile flow during cardiopulmonary bypass preserves postoperative microcirculatory perfusion irrespective of systemic hemodynamics

N.J. Koning, A.B.A. Vonk, L.J.M. van Barneveld, A. Beishuizen, B. Atasever, C.E. van den Brom, C. Boer

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Abstract

Background: The onset of nonpulsatile cardiopulmonary bypass is known to deteriorate microcirculatory perfusion, but it has never been investigated whether this may be prevented by restoration of pulsatility during extracorporeal circulation. We therefore investigated the distinct effects of nonpulsatile and pulsatile flow on microcirculatory perfusion during on-pump cardiac surgery.

Methods: Patients undergoing coronary artery bypass graft surgery were randomized into a nonpulsatile (n=17) or pulsatile (n=16) cardiopulmonary bypass group. Sublingual mucosal microvascular perfusion was measured at distinct perioperative time intervals using sidestream dark field imaging, and quantified as the level of perfused small vessel density and microvascular flow index (vessel diameter < 20 μm). Microcirculation measurements were paralleled by hemodynamic and free hemoglobin analyses.

Results: The pulse wave during pulsatile bypass estimated 58 ± 17 % of the baseline blood pressure waveform. The observed reduction in perfused vessel density during aorta cross-clamping was only restored in the pulsatile flow group and increased from 15.5 ± 2.4 to 20.3 ± 3.7 mm/mm^2 upon intensive care admission (P<0.01). The median postoperative microvascular flow index was higher in the pulsatile group (2.6 (2.5-2.9)) than in the nonpulsatile group (2.1 (1.7-2.5); P=0.001). Pulsatile flow was not associated with augmentation of free hemoglobin production and was paralleled by improved oxygen consumption from 70 ± 14 to 82 ± 16 ml/min/m^2 (P=0.01) at the end of aortic cross-clamping.

Conclusion: Pulsatile cardiopulmonary bypass preserves microcirculatory perfusion throughout the early postoperative period, irrespective of systemic hemodynamics. This observation is paralleled by an increase in oxygen consumption during pulsatile flow, which may hint towards decreased microcirculatory heterogeneity during extracorporeal circulation and preservation of microcirculatory perfusion throughout the perioperative period.

Introduction

Earlier studies by our group and others have shown that cardiopulmonary bypass (CPB) with nonpulsatile flow is associated with a decrease in capillary density and microvascular flow, most likely as a result of hemodilution, hypothermia, inflammation and endothelial dysfunction [1-3]. Loss of microcirculatory perfusion during extracorporeal circulation may contribute to the development of organ dysfunction and has been indicated as independent predictor of outcome [4,5]. Although restoration of pulsatile flow during cardiopulmonary bypass could be beneficial for postoperative outcome, there is an ongoing debate about the mechanisms underlying the favorable effects of pulsatility during extracorporeal circulation [6]. Reported advantages of pulsatile cardiopulmonary bypass include preserved renal function, reduced liver damage, reduced levels of endotoxins, less need for inotropics and a lower mortality after surgery [7-9].

There are only limited data available about the implications of nonpulsatile and pulsatile flow on microcirculatory perfusion during CPB. A recent cross-over study by Elbers et al. showed no difference in microcirculatory perfusion after ten minutes of either pulsatile or nonpulsatile CPB, but their conclusions were limited by the study design [10]. In contrast, laser Doppler flow or gastric tonometry measurements indirectly indicated that pulsatility may preserve microvascular perfusion [11-13]. At similar mean arterial pressures, pulsatile flow was associated with a 10-15% more energy equivalent pressure than nonpulsatile flow [14], which has previously been suggested to be a prerequisite to overcome the critical capillary closing pressure [15]. Under experimental conditions, nonpulsatile flow further resulted in reduced endothelial shear stress that may contribute to endothelial dysfunction and increased microvascular resistance [16]. Others showed that distinct flow conditions affect endothelial nitric oxide synthase expression, prostacyclin and pro-inflammatory protein production and the induction of vascular adhesion molecules [17,18]. In particular, pulsatile flow increases endothelial nitric oxide production as compared to nonpulsatile flow [19,20].

It is questioned whether pulsatile flow is indeed associated with preserved microvascular perfusion as compared to nonpulsatile flow during extracorporeal circulation. We therefore investigated whether pulsatile flow preserves microvascular perfusion during and after cardiopulmonary bypass in relatively healthy patients undergoing coronary artery bypass graft surgery. Direct microvascular perfusion measurements of the sublingual mucosa were performed during the intraoperative and postoperative period using Sidestream Dark Field (SDF) imaging, a video technique that allows direct microscopic observation of the microcirculation [21]. We further investigated whether our observations were associated with alterations in blood damage or could be linked to systemic hemodynamic alterations.

Methods

Study population

The local Human Subjects Committee approved this single center, prospective, clinical study and written informed consent was obtained from all subjects. The study group included 34 patients undergoing elective coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass (CPB). Previous heart surgery, emergency surgery, insulin-dependent diabetes mellitus and a body mass index >30 kg/m^2 were considered as exclusion criteria. Clopidogrel was stopped at five days preoperatively, whereas acetylsalicylic acid was continued. Patients were randomized into the nonpulsatile flow or pulsatile flow study group by envelope drawing.

Anesthesia protocol

On the day of surgery, coronary patients received their usual early morning dose of antianginal medication and lorazepam (5 mg), whereas no diuretics were given. Anesthesia consisted of sufentanil (3-7 µg/kg) with pancuronium bromide (0.1 mg/kg) and midazolam (0.1 mg/kg) and was maintained by continuous propofol infusion (5-15 ml/h). Ventilation parameters were: 8-10 ml/kg tidal volume, 4-5% end-tidal CO_2, 45% O_2-air mixture, positive end-expiratory pressure of 5 cm H_2O. All patients received dexamethasone (1 mg/kg) and cefazolin (1000 mg). In 16 patients, a thermodilution pulmonary artery catheter was placed based on the decision of the anesthesiologist.

Cardiopulmonary bypass

An S3 heart-lung machine with heater-cooler device (Stöckert Instrumente GMBH, Munich, Germany) and centrifugal pump (Sarns, Terumo Europe NV, Leuven, Belgium) were used for CPB. The extracorporeal circuit consisted of a heparin coated polyvinyl tubing system with a hollow fiber oxygenator and arterial line filter (Affinity, Medtronic, Minneapolis, MN, USA), a soft shell venous reservoir (MVR 1600, Medtronic) and a cardiotomy reservoir (Intercept cardiotomy, Medtronic). The circuit was primed with 1000 ml modified fluid gelatin (Braun Melsungen AG, Germany), 100 ml mannitol (20%, Baxter BV, Utrecht, Netherlands), 50 ml sodium bicarbonate (8.4% Braun Melsungen AG) and 50 ml lactated Ringer's solution (Baxter BV), containing 1000 mg cefalozin (Eli Lilly Nederland BV, Nieuwegein, Netherlands) and 5000 IU bovine heparin. After heparin (300 IU/kg) administration, the arterial cannula was placed in the ascending aorta and the other (two stage) venous cannula (36 French) in the right atrium. Venting of the left ventricle was achieved by an aortic root-cannula. CPB (34°C; 2.2-3.0 l/min/m^2) was initiated when the activated clotting time exceeded 480 s. Myocardial protection was achieved by 4°C crystalloid cardioplegia solution (St. Thomas). Patients were weaned from CPB when rectal temperature estimated 36°C. Heparin was reversed with protamine in a 1:1 fashion.

Pulsatile or nonpulsatile flow

In the pulsatile flow group, the flow character of the centrifugal pump was set to pulsatile during aortic cross-clamp time. The pump used a standard internal algorithm to generate pulsatility with a frequency of 60 per minute. Area under the curve analysis was performed on cycles of the arterial blood pressure waveform to provide a relative quantification of the strength of the arterial pressure wave as measured in the radial artery. Comparisons were made between cycles before CPB and cycles during aortic cross-clamping. During two phases of extracorporeal circulation without aortic cross-clamp, the centrifugal pump was set to the nonpulsatile mode, though surgeons intended to preserve flow through the heart, leading to varying pulsatility at these time points (T2 and T5). In the nonpulsatile flow group standard centrifugal pump settings were used resulting in a continuous, nonpulsatile blood flow.

SDF-imaging

Sublingual mucosal microcirculation measurements were performed during surgery and at the intensive care unit (ICU) using Side Dark Field (SDF) imaging (MicroScan, Microvision Medical, Amsterdam, Netherlands). SDF and its predecessor, orthogonal polarization spectral (OPS) imaging, have been used extensively in clinically assessing microcirculatory perfusion [1-5,10,22,23] SDF demonstrated improved capillary contrast and quality in comparison to the earlier developed orthogonal polarization spectral (OPS) imaging technique in a validation study [21]. Briefly, SDF imaging is based on non-invasive hand-held video microscopy with a light guide placed on organ surfaces for direct microscopic observation of the microcirculation [21]. A 5 x magnifying lens (field area of 1 mm^2) projected the image to a video camera inside the device. Microvessel images were observed on a monitor with a final magnification of 350x and recorded on digital tapes for off-line computer analysis. The images were made after induction of anesthesia (T1; preop), after onset of CPB before cross-clamping the aorta (T2; CPB I), early after cross-clamping the aorta (T3; AoX I), before removing the aortic cross-clamp (T4; AoX II), after removing the aortic cross-clamp (T5; CPB-II), after weaning from CPB while under anesthesia (T6; post-CPB) and in the first hour after ICU admission (T7; ICU). At each time point, three SDF sequences of 10 seconds were recorded. Each video clip was analyzed with automatic vascular analysis software (AVA 3.0, Microvision Medical, Amsterdam), according to the recommendations by De Backer et al. [24]. Analyses were performed by one of the authors, and one third of the video clips were randomly reviewed by two of the other authors in order to check the inter-rater agreement. All vessels were identified manually, which enabled the software to calculate the total vessel density (TVD) in mm/mm^2. Subsequently, small vessels relevant for oxygen exchange as reflected by a diameter < 20 µm were scored separately using the following classifications: no flow, sluggish flow, intermittent flow and continuous flow. For the calculation of the perfused vessel density (PVD) in mm/mm^2, the software considered vessels scored with intermittent

or no flow as nonperfused, whereas continuous or sluggish scores were regarded as perfused vessels. For determination of the microvascular flow index (MFI), the screen was divided in four quadrants. The same standard classification as described above was used to explain the predominant flow pattern for an entire quadrant. No flow, sluggish flow, intermittent flow and continuous flow represented scores from 0 to 3, respectively. The mean score of the four quadrants represented the MFI. The inter- and intra-rater agreement for the microvascular flow index has been shown to be 90% and 85%, respectively [22].

Blood samples

Plasma free hemoglobin was assessed before extracorporeal circulation and after nonpulsatile or pulsatile cardiopulmonary bypass (Haemoscan BV, Groningen, The Netherlands). Blood samples drawn upon intensive care unit admission were analyzed for leukocyte count, C-reactive protein (CRP), and creatinine according to routine laboratory procedures. The release of IL-6, vascular endothelial growth factor (VEGF) and tumor necrosis factor α (TNF-α) was determined at abovementioned time points using commercially available ELISA kits (BioLegend's ELISA MAX™ Deluxe Set, R&D systems). ELISA was performed following the instructions of the manufacturer. Cardiac index was determined by thermodilution cardiac output measurements (T=1, T=6, T=7) and by CPB flows (T=3, T=4) in a subgroup of patients (nonpulsatile CPB: n=7, pulsatile CPB: n=9). The oxygen consumption (VO_2i) and delivery index (DO_2i) and oxygen extraction ratio (VO_2i /DO_2i) were calculated at T=3 (AoX start) and T=4 (AoX end) using the cardiac index derived from CPB flow in combination with arterial and mixed venous blood gas values. The following formulas were used: $DO_2i = C_aO_2$ x cardiac index; $VO_2i = (C_aO_2 - C_vO_2)$ x cardiac index; C_aO_2 (arterial content O_2) = hemoglobin x 1.36 / 0.6206 x S_aO_2 x 10 + P_aO_2 x 0.0031; C_vO_2 = hemoglobin x 1.36 / 0.6206 x S_vO_2 x 10 + P_vO_2 x 0.0031.

Statistical analysis

Data were analyzed using a SPSS statistical software package (version 17.0). All values are expressed as mean ± standard deviation or median with interquartile range (IQR). Repeated-measures (RM) ANOVA was performed to analyze between-group effects of parameters with multiple time points (mean arterial pressure, cardiac index, TVD and PVD). Changes from baseline values and changes during cross-clamp time for temperature, hemoglobin, hematocrit, VO_2i, DO_2i and oxygen extraction ratio were analyzed by a paired T-test, between group effects were tested by a Student's T-test. Differences in MFI were tested for individual time points using a Mann Whitney U test. $P < 0.05$ was considered as statistically significant.

Results

Intraoperative and postoperative hemodynamics

After exclusion of one patient due to a large variation in intraoperative and microcirculatory data, the study population included 33 patients for final analysis. Figure 1 represents typical examples of arterial blood pressure waveforms for nonpulsatile and pulsatile flow in individual patients. Area-under-curve analysis of the arterial blood pressure waveforms in eight subjects revealed a pulse wave during the pulsatile flow mode with an area under the curve of 58 ± 17 % of the pulse wave during systemic circulation before cardiopulmonary bypass. In the nonpulsatile flow mode, the area under the curve estimated 0 ± 0 % from baseline (n=3).

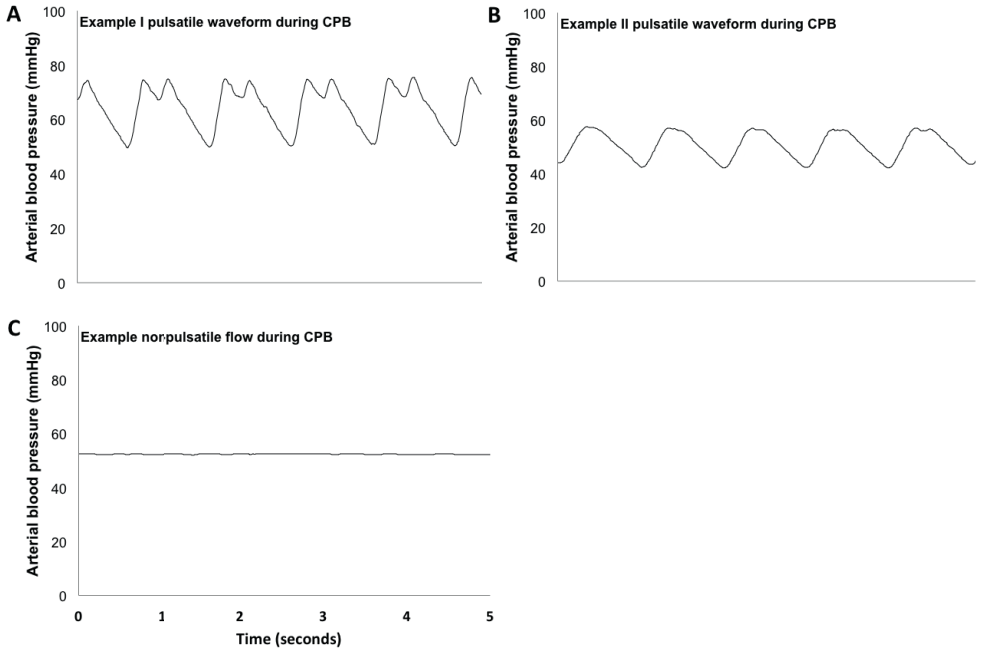

Figure 1 Typical individual examples of arterial blood pressure waveforms measured in the arteria radialis during aortic cross-clamping by a centrifugal pump in the pulsatile (panels A and B) and nonpulsatile (panel C) flow mode.

Patient characteristics are presented in Table 1.

Table 1 Patient characteristics

Description	Nonpulsatile flow	Pulsatile flow
N	17	16
Age (years)	65 ± 7	67 ± 11
Males [n] (%)	14 / 17 (82%)	10 / 16 (63%)
BSA (m^2)	2.1 ± 0.2	1.9 ± 0.2
Diabetes Mellitus II [n] (%)	2 / 17 (12%)	4 / 16 (25%)
Antihypertensive treatment [n] (%)	14 / 17 (82%)	14 / 16 (88%)
CPB time (min)	98 ± 28	106 ± 20
Cross-clamp time (min)	63 ± 22	72 ± 14
Anastomoses [median (IQR)]	3 (3- 4)	3 (3- 5)

Values are represented as mean ± standard deviation. BSA = Body surface area, CPB = cardiopulmonary bypass, IQR = interquartile range.

Repeated measures ANOVA revealed no differences in mean arterial blood pressure values (Figure 2A; P=0.35) and cardiac index (Figure 2B; P=0.25) over time between nonpulsatile and pulsatile flow groups.

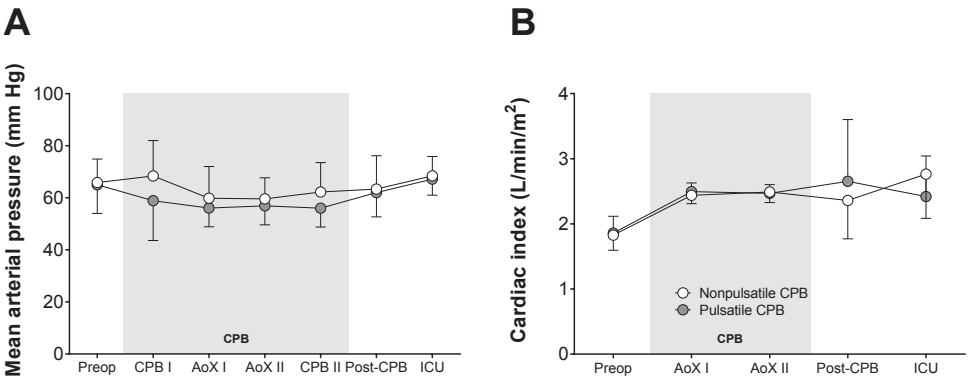

Figure 2 Mean arterial blood pressure (panel A) and cardiac index (panel B) for nonpulsatile (white circles) and pulsatile (grey circles) cardiopulmonary bypass. Blood pressure and cardiac index were similar for both groups over time. The grey rectangles in the background indicate the period on cardiopulmonary bypass. AoX = aortic cross-clamping; Preop = after induction of anesthesia; CPB = cardiopulmonary bypass; ICU = intensive care unit.

Cardiopulmonary bypass was associated with a decrease in body temperature, hemoglobin and hematocrit, but we found no differences between groups (Table 2).

Table 2 Intra- and postoperative temperature, hemoglobin and hematocrit

		Preop	AoX I	AoX II	ICU
		T1	T3	T4	T7
Temperature (°C)	**NP**	36.3 ± 0.5	35.3 ± 0.7 #	35.4 ± 0.6 #	-
	P	36.2 ± 0.6	34.9 ± 0.7 #	35.2 ± 0.6 #	-
Hemoglobin (mmol/l)	**NP**	8.1 ± 0.6	5.4 ± 0.7 #	5.6 ± 0.5 #‡	7.0 ± 0.5 #
	P	7.9 ± 1.0	5.3 ± 0.9 #	5.6 ± 0.9 #‡	6.8 ± 0.8 #
Hematocrit	**NP**	0.39 ± 0.03	0.27 ± 0.04 #	0.27 ± 0.03 #‡	0.32 ± 0.03 #
	P	0.39 ± 0.05	0.26 ± 0.05 #	0.28 ± 0.04 #	0.32 ± 0.04 #

Values represent mean ± standard deviation. NP = nonpulsatile (n=17); P = pulsatile (n=16). # P<0.05 versus T1, ‡ P<0.05 versus T3.

In a subgroup of patients with a pulmonary artery catheter we observed an immediate decrease in the oxygen consumption index in both groups upon the start of extracorporeal circulation. Figure 3 shows the oxygen delivery index (DO_2i; panel A), oxygen consumption index (VO_2i; panel B) and oxygen extraction ratio (panel C) for the nonpulsatile and pulsatile groups at the beginning (AoX start) and end of aortic cross-clamping (AoX end). There were no alterations in DO_2i in both groups, whereas the VO_2i (69.8 ± 13.8 vs. 82.4 ± 15.6 ml O_2/m^2/min; paired T-test P=0.01) and oxygen extraction ratio (0.23 ± 0.05 vs. 0.26 ± 0.04; paired T-test P=0.035) were increased at the end of aortic cross-clamping (AoX end) in the pulsatile flow group when compared to baseline (AoX start).

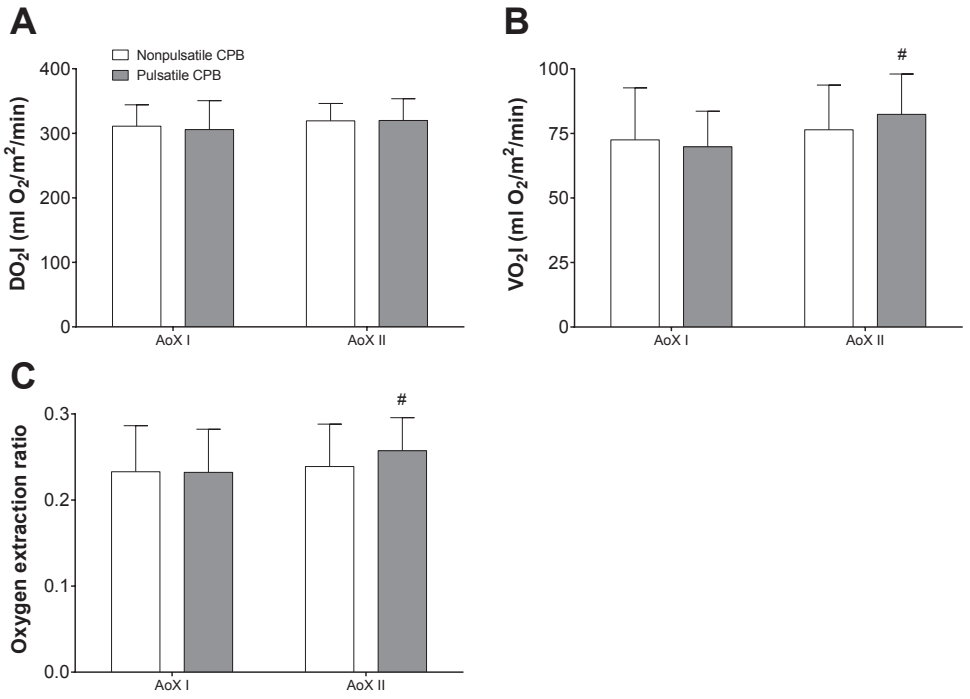

Figure 3 Oxygen delivery index (DO_2I; panel A), oxygen consumption index (VO_2I; panel B) and oxygen extraction ratio (panel C) for nonpulsatile (n=17; white) and pulsatile (n=16; grey) flow at the beginning (AoX I) and end (AoX II) of aortic cross-clamping. #P<0.05 vs AoX I.

Microcirculatory perfusion

Figure 4 shows the sublingual total microcirculatory vessel density (TVD; panel A) and the perfused microcirculatory vessel density (PVD; panel B) for vessels smaller than 20 μm. In both nonpulsatile and pulsatile groups, the TVD reduced by 20% during aortic cross-clamping (AoX end) as compared to baseline (Preop). The decline in TVD tended to be transient in the pulsatile flow group, but this restoration was not significantly different from the laminar flow group (repeated measures ANOVA; P=0.13). The observed reduction in perfused vessel density (PVD) during aorta cross-clamping was only restored in the pulsatile flow group and increased from 15.5 ± 2.4 to 20.3 ± 3.7 mm/mm^2 upon intensive care admission in this group (repeated measures ANOVA; P=0.02). The perfused vessel density remained at a stable level in the nonpulsatile flow group until ICU admission.

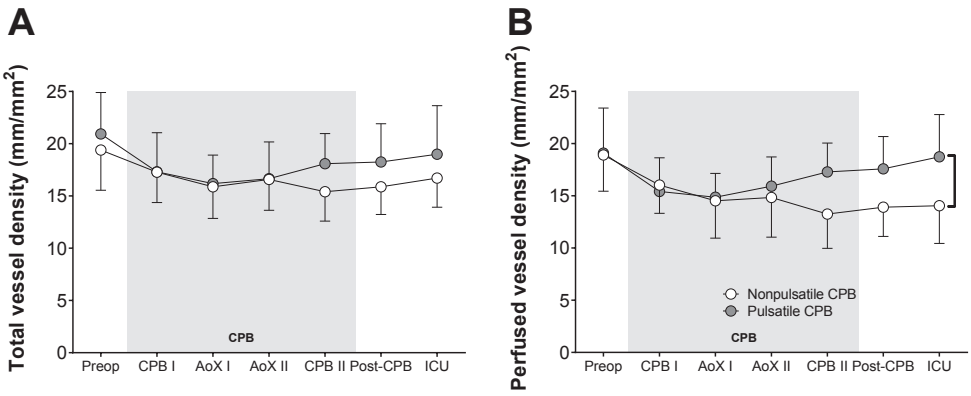

Figure 4 Total small vessel density (panel A) and perfused small vessel density (panel B) for nonpulsatile (white circles; n=17) and pulsatile (grey circles; n=16) cardiopulmonary bypass (CPB). The grey rectangles in the background indicate the period on cardiopulmonary bypass. After aortic cross-clamping (AoX) the perfused vessel density decreased in both groups, but only restored in the pulsatile group after removal of the aortic clamp (P=0.02 between groups; repeated measures ANOVA). AoX = aortic cross-clamping; Preop = after induction of anesthesia; CPB = cardiopulmonary bypass; ICU = intensive care unit. Parentheses in panel B indicate P<0.05 between groups.

The effect of pulsatile flow on the microvascular flow index (MFI) is depicted in Figure 5 for small vessels. Starting from the end of aortic cross-clamping (AoX end), the MFI significantly restored in the pulsatile group when compared to nonpulsatile flow. The median postoperative microvascular flow index was higher in the pulsatile group (2.6 (2.5-2.9)) than in the nonpulsatile group (2.1 (1.7-2.5); P=0.001).

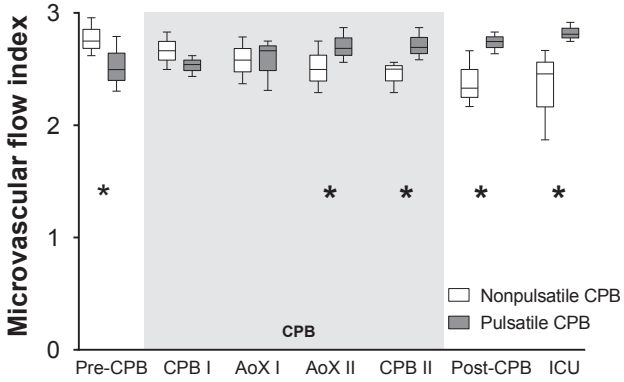

Figure 5 Microvascular flow index, where 0 to 3 on the y-axis corresponds with no flow, intermittent flow, sluggish flow and continuous flow, respectively. The grey rectangles in the background indicate the period on cardiopulmonary bypass. Pulsatile flow preserved the microvascular flow index as compared to nonpulsatile flow. AoX = aortic cross-clamping; Pre-CPB = after induction of anesthesia; CPB = cardiopulmonary bypass; ICU = intensive care unit. *P<0.05 between groups.

Blood samples

Cardiac surgery with cardiopulmonary bypass induced an equal increase in plasma free hemoglobin in the nonpulsatile (from 0.12 ± 0.04 mg/l to 0.22 ± 0.15 mg/l (n=15); P=0.021) and pulsatile group (from 0.10 ± 0.05 mg/l to 0.23 ± 0.12 mg/l (n=14); P=0.004). There were no differences in IL-6 (126.8 ± 78.7 pg/ml (n=15) vs. 122.0 ± 73.9 pg/ml (n=12), P=0.74) or VEGF (7.8 ± 8.0 pg/ml (n=14) vs. 6.3 ± 8.7 pg/ml (n=12), P=0.92) upon intensive care admission after nonpulsatile and pulsatile flow, respectively. TNF-α levels tended to be elevated in the pulsatile flow group (21.3 ± 13.5 pg/ml; n=13) compared to the nonpulsatile flow group (12.6 ± 7.8 pg/ml (n=15); P=0.054.) Upon intensive care admission, there were no differences in leukocyte count (13.0 ± 4.9·10^9/l (n=16) vs. 14.9 ± 7.8·10^9/l (n=16); P=0.43), C reactive protein (91.1 ± 57.1 mg/l (n=15) vs. 75.9 ± 44.3 mg/l (n=14); P=0.43) or creatinine (82.4 ± 20.5 µmol/l (n=17) vs. 78.8 ± 19.0 µmol/l (n=16); P=0.61) between nonpulsatile and pulsatile flow groups, respectively.

Clinical outcome

None of the subjects developed postoperative renal failure, myocardial infarction or cerebrovascular accidents. In both nonpulsatile and pulsatile CPB groups, 4 patients developed de-novo atrial fibrillation. There was no mortality in the first 30 days after surgery and all patients were discharged from the intensive care unit within 24 hours after surgery.

Discussion

Nonpulsatile flow during extracorporeal circulation was associated with sustained reduction in microcirculatory density and perfusion upon intensive care admission. In contrast, patients exposed to pulsatile flow showed a fast recovery of microcirculatory perfusion after weaning from cardiopulmonary bypass. This study is the first to demonstrate that pulsatile flow during extracorporeal circulation preserves microcirculatory perfusion as measured by sublingual sidestream dark field (SDF) imaging of the microcirculation. Systemic hemodynamic parameters were not explanatory for the preservation of microcirculatory perfusion under pulsatile conditions. During cross-clamp time, pulsatile flow was associated with a slightly improved oxygen consumption and extraction rate, whereas nonpulsatile flow did not improve these parameters. Pulsatile and nonpulsatile CPB increased hemolysis equally. Despite better recovery of the microcirculation in the pulsatile group, no differences in clinical outcome parameters between pulsatile and nonpulsatile flow groups were found. However, the current study was not powered to detect differences between groups in clinical outcome, and we only included relatively healthy patients undergoing low risk cardiac surgery. We hypothesize that the magnitude of our current findings may be enlarged in cardiosurgical procedures with a higher predicted mortality. To place our investigation in perspective, we showed that preservation of the physiological situation during extracorporeal circulation by means of pulsatile flow seems to markedly reduce microcirculatory recovery time, as it was previously shown that restoration of microvascular heterogeneity usually takes 6 hours after surgery [3].

The beneficial effects of pulsatile flow on microcirculatory perfusion are in agreement with findings by others who showed that pulsatility reduced markers of endothelial damage and improved gastric mucosal oxygenation and tonometry [12,13]. Here we confirm these findings by direct measurements of microcirculatory perfusion. In contrast, Elbers et al. recently showed that short-term pulsatile CPB was not beneficial for microcirculatory perfusion when compared to nonpulsatile flow [10]. This study focused especially on mechanical consequences of pulsatility rather than the effect of pulsatile flow on microcirculatory perfusion following surgery. Due to an average pulse pressure of 7 mmHg there was no true loss of pulsatility in their control group. Moreover, the crossover design of the study, which has earlier been criticized by Undar et al. [25], limits their conclusions and may further hinder comparison with our results.

An interesting study published by Onorati et al. showed promising results for pulsatile flow in patients undergoing CABG surgery with intra-aortic balloon pump (IABP) support [26]. Postoperative renal function, liver function, lung function and hemostasis and endothelial markers were all favorable in the pulsatile group. However, in agreement with our findings there were no differences in general inflammatory markers like IL-6 and TNF-α between

nonpulsatile and pulsatile flow [26]. Although the IABP-created pulse is different from the pulse generated by a centrifugal pump, the beneficial clinical results in IABP patients suggest that striving for pulsatility during CPB may be justified. Since IABP support is absent in the majority of CABG patients, pulsatility created in the extracorporeal system may provide an important alternative for this group of patients.

Preservation of endothelial function and reduction of inflammatory pathways are currently the most widely used concepts to explain the beneficial effects of pulsatile flow during CPB. It has indeed been shown that the absence of pulsatility is associated with capillary fall out, endothelial damage, endotoxin translocation and the production of cytokines, endothelin-1, lactate and catecholamines [9,27-29]. Interestingly, Sezai et al. showed that the increased production of endothelin-1 with nonpulsatile flow starts no earlier then after weaning from cardiopulmonary bypass, simultaneously with impairment of microcirculatory recovery as shown in our investigation [28]. However, we did not focus on vascular physiological mechanisms that may explain the beneficial effects of pulsatility. Future studies should therefore focus on the underlying mechanism of the beneficial effects of pulsatile flow during CPB. Most of the inflammatory markers that were determined in our investigation did not differ between both groups, and were therefore not explanatory for the observed differences in the restoration of microcirculatory flow.

Systemic hemodynamic changes were not of influence on microcirculatory perfusion after nonpulsatile or pulsatile CPB. This is in agreement with De Backer et al., who earlier showed that microcirculatory perfusion is independent of hemodynamic parameters [23]. Kindig et al. showed that rats with chronic heart failure developed a decreased proportion of skeletal muscle vessels supporting red blood cell flux [30]. This reduction in capillary perfusion is most probably attributed to an increased arteriolar tone, venous congestion and reduced cardiac output. In contrast, reduced capillary perfusion during extracorporeal circulation is paralleled by an increase in cardiac output and reduction of systemic vascular resistance. From our findings it might be suggested that the acute onset of hemodilution, activation of inflammatory and coagulation pathways and pulseless flow are more explanatory for the reduction in microcirculatory perfusion than changes in cardiac output. The findings of Kindig et al. and our investigation demonstrate the diversity in physiological mechanisms that may lead to a change in capillary perfusion [30].

It has been shown that sepsis is associated with an increased heterogeneity of microcirculatory perfusion [31], which may subsequently lead to impairment in oxygen extraction [32,33]. Moreover, heterogeneous perfusion of the microcirculation, reflected by a combination of blood flow cessation and hyperdynamic vessels, is deleterious for tissue oxygenation [34,35]. De Backer and coworkers found that an increase in the heterogeneity of microcirculatory flow is also present during CPB [3] In line with this, our group showed earlier that the onset of CPB is associated with decreased perfused capillary density, increased venular blood velocity

and increased microvascular hemoglobin oxygen saturation [1]. Altogether, these findings suggest an increase in flow through a reduced number of capillaries, resulting in a decrease in erythrocyte oxygen offloading during CPB. In the current study we demonstrated that, after pulsatile CPB, perfused vessel density is recovered whereas oxygen consumption is improved during cross-clamp time. A recent study by Karaci et al. also demonstrated that pulsatile flow is indeed associated with improvements in oxygen consumption and extraction ratio when compared to nonpulsatile conditions [36]. These and our findings hint towards the idea that pulsatile CPB is beneficial for recovering from microcirculatory heterogeneity, and consequently reduces the risk for a reduced oxygen extraction.

Finally, we showed that the differences in perfused vessel density between groups are the most pronounced after CPB, and were undetectable during aortic cross-clamping. These findings might suggest that pulsatile flow has no direct effect on microcirculatory perfusion, but induces delayed physiological mechanisms that contribute to preservation of microcirculatory flow after surgery. The hypothesis that pulsatile energy is used to overcome a critical capillary closing pressure during CPB seems therefore unlikely [14,15].

Increased hemolysis is a possible negative effect of pulsatile flow due to higher peak pressures in the extracorporeal circuit [8,37]. However, we did not find differences in post-bypass plasma free hemoglobin levels between the nonpulsatile and pulsatile groups. The concomitant negative effects of blood damage may reverse the beneficial effects of pulsatile cardiopulmonary bypass, which could be explanative for the absent advantage of pulsatility as reported by some authors.

Our study is limited by the absent calculation of the Energy Equivalent Pressure (EEP). Although EEP is regarded as the standard method for quantification of pulsatility we showed that complete absence of pulsatility during aortic-cross clamping is associated with disturbed microcirculatory perfusion when compared to pulsatile CPB. In addition, we demonstrated that our pulsatile modality of the heart-lung machine was indeed associated with radial blood pressure waveforms with an area under the curve of about 60% of the baseline systemic arterial blood pressure waveform.

Furthermore, one may argue whether the sublingual mucosa is representative for vital organs that may be affected by the deleterious effects of cardiopulmonary bypass on microcirculatory perfusion. Changes in microcirculatory flow in the sublingual mucosa are however well correlated with alterations in gastric and intestinal beds, as shown by pCO_2 measurements, OPS and SDF-imaging and colored-microsphere blood flow measurements [38-43]. In addition, the sublingual mucosa shares a common embryologic origin with the intestinal mucosa. Although extrapolation of the current data may be limited to splanchnic microcirculatory beds, these are of particular importance in maintenance of barrier integrity to prevent endotoxin translocation [9]. Moreover, sublingual SDF and OPS-imaging studies have demonstrated that persistent sublingual microcirculatory disturbances are associated

with poor outcome of critical ill patients [4,5]. Also, the sublingual microcirculation has been used in previous studies during cardiac surgery [1-3].

In conclusion, pulsatile flow by a centrifugal pump during aortic cross-clamp time is associated with preservation of microvascular perfusion in the early postoperative period, irrespective of systemic hemodynamics. While both nonpulsatile and pulsatile groups showed a marked decrease in perfused capillary density during cardiopulmonary bypass, microcirculatory perfusion only returned to preoperative baseline values in patients undergoing pulsatile cardiopulmonary bypass. Our findings were independent of systemic inflammatory blood parameters, whereas only pulsatile flow improved oxygen consumption during cross-clamping. These findings support the hypothesis that pulsatile flow may attenuate microcirculatory heterogeneity during extracorporeal circulation in comparison to nonpulsatile flow, although the underlying mechanism remains unknown. Although we found no differences in clinical outcome in our low-risk study population that underwent short-lasting, uncomplicated cardiosurgical procedures, further studies should elaborate whether preservation of microcirculatory perfusion in the early postoperative period due to intraoperative pulsatile flow is additionally associated with improved outcome in patients undergoing more complicated cardiac surgery.

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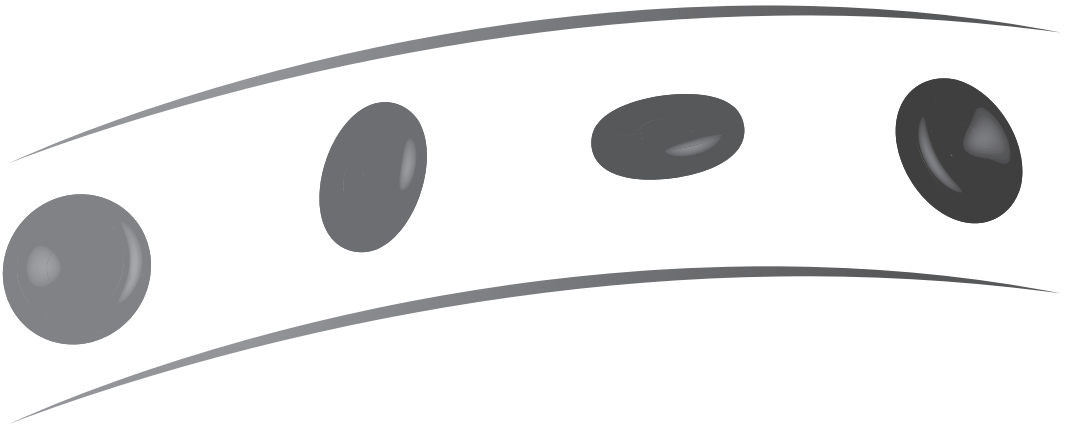

CHAPTER 5

Systemic microvascular shunting through hyperdynamic capillaries after acute physiological disturbances following cardiopulmonary bypass

N.J. Koning, L.E. Simon, P. Asfar, C. Baufreton, C. Boer.

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Abstract

Background: Previously we showed that cardiopulmonary bypass during cardiac surgery is associated with reduced sublingual microcirculatory perfusion and oxygenation. It has been suggested that impaired microcirculatory perfusion may be paralleled by increased heterogeneity of flow in the microvascular bed, possibly leading to arteriovenous shunting. Here we investigated our hypothesis that acute hemodynamic disturbances during extracorporeal circulation indeed lead to microcirculatory heterogeneity with hyperdynamic capillary perfusion and reduced systemic oxygen extraction.

Methods: In this single-center prospective observational study, patients undergoing cardiac surgery with (n=18) or without (n=13) cardiopulmonary bypass (CPB) were included. Perioperative microcirculatory perfusion was assessed sublingually with sidestream dark field imaging and recordings were quantified for microcirculatory heterogeneity and hyperdynamic capillary perfusion. The relationship with hemodynamic and oxygenation parameters was analyzed.

Results: Microcirculatory heterogeneity index increased substantially after onset of CPB (0.5 [0.0-0.9] to 1.0 [0.3-1.3]; P=0.031) but not during off-pump surgery. Median capillary red blood cell (RBC) velocity increased intraoperatively in the CPB group only (1600 [913-2500 µm/s] versus 380 [190-480 µm/s]; P<0.001), with 31% of capillaries supporting high RBC velocities (>2000 µm/s). Hyperdynamic microcirculatory perfusion was associated with reduced arteriovenous oxygen difference and systemic oxygen consumption during and after CPB.

Conclusion: The current study provides the first direct human evidence for a microvascular shunting phenomenon through hyperdynamic capillaries following acute physiological disturbances after onset of cardiopulmonary bypass. The hypothesis of impaired systemic oxygen offloading caused by hyperdynamic capillaries was supported by reduced blood arteriovenous oxygen difference and low systemic oxygen extraction associated with cardiopulmonary bypass.

Introduction

The microcirculation is an important end organ in the pathophysiology of critical illnesses [1], and remains to date a black box in clinical practice [2]. Under normal circumstances, tissue microcirculatory perfusion is largely homogenous and adaptive to serve a balance between oxygen supply and demand. However, diseases like sepsis or ischemia/reperfusion injury are paralleled by microcirculatory disturbances with increased regional heterogeneity of perfusion [3,4], characterized by a combination of capillaries with low blood flow and capillaries with extremely high blood velocities [5,6].

Microcirculatory heterogeneity of flow leads to impaired tissue oxygen extraction [6,7] and is associated with unfavorable outcome in multiple patient populations [8-11]. Hyperdynamic capillary blood flow theoretically causes inefficient red blood cell (RBC) offloading, and therefore provides a functional arteriovenous shunt of oxyhemoglobin [12].

A specific feature of cardiopulmonary bypass (CPB) is an instant onset of a non-physiological, high flow, low resistance circulation, similar to the hemodynamic features observed in septic shock [13]. Moreover, we previously showed that acute disturbances in microcirculatory perfusion take place after onset of cardiopulmonary bypass [1]. Reduced systemic vascular resistance in combination with a high systemic blood flow and acute hemodilution might predispose for hyperdynamically perfused capillaries.

We hypothesized that acute cardiopulmonary bypass-induced hemodynamic disturbance leads to increased microcirculatory heterogeneity, with simultaneous hyperdynamically perfused capillaries and altered oxygen-offloading parameters. In order to investigate our hypothesis, we performed sublingual microcirculatory video microscopy in patients undergoing cardiopulmonary bypass. Patients undergoing cardiac surgery without cardiopulmonary bypass served as control group. As the influence of systemic hemodynamics on the microcirculation cannot be ruled out we also focused on the relationship between the systemic and the microcirculation hemodynamics.

Methods

Study design

The present investigation was a single-center, prospective observational study performed in the operation theater and at the intensive care unit of the VU University Medical Center. The study was executed in accordance with the Declaration of Helsinki and was approved by the University Human Subjects Committee. Written informed consent was obtained from all patients before inclusion. Patients between 40 and 85 years of age scheduled for elective coronary bypass graft surgery (CABG) with (n=18) or without (n=13) cardiopulmonary bypass were eligible for study inclusion. Exclusion criteria were re-operations, emergency operations, insulin dependent diabetes mellitus, body mass index (BMI) >35 kg/m^2 and a preoperative hemoglobin level <5.5 mmol/l. No preoperative selection on patient characteristics was applied for allocation to either off-pump or on-pump surgery. The use of cardiopulmonary bypass was based on the operating schedule, as all off-pump surgical procedures were performed by a single cardiothoracic surgeon. Study inclusion took place only after patients were allocated to a surgical techniques and therefore did not influence group composition.

Anesthesia

Anesthesia was induced in all patients with 1 to 3 µg/kg intravenous sufentanil, 0.1 mg/kg midazolam and 0.1 mg/kg pancuronium bromide, whereas maintenance occurred with a continuous infusion of propofol (200 to 400 mg/h). Patients were ventilated with tidal volumes of 6 to 8 ml/kg, and a frequency set to obtain an end-tidal CO_2 concentration of between 4 and 5%, and with a fraction of inspired oxygen of 0.45. A positive end-expiratory pressure of 5 cm H_2O was applied. After induction of anesthesia, patients received 1 mg/kg dexamethasone and 1000 mg cefazolin. A pulmonary artery catheter was inserted in all patients as part of the study protocol. Administration of vasoactive medication (nitroglycerine and dopamine on continuous infusion or bolus administration of phenylephrine, all if necessary) was based on the decision of the anesthesiologist.

CPB group

An S5 heart-lung machine (Stöckert Instrumente GMBH, Munich, Germany) with a nonpulsatile centrifugal pump (Delphin, Terumo Europe NV, Leuven, Belgium) with heparin coated extracorporeal circuit (Medtronic, Minneapolis, MN, USA) primed with a total of 1400 mL non-blood priming solution were used for CPB as previously described [14].

Heparin was administered (300 IU/kg) before cannulation of the ascending aorta and the right atrium to achieve an activated clotting time of 480 seconds. The blood flow during mild hypothermic (34-35°C) CPB was kept between 2.2 and 3.0 l/min/m^2. Target mean arterial pressure (MAP) was 60 mm Hg. A cell-saver suction device was used to retransfuse washed

pericardial shed blood. After weaning from the extracorporeal circulation, protamine was administered to reverse heparin in a 1:1 ratio.

Off-pump group

Before grafting, an initial heparin dose of 300 IU/kg was administered, and the ACT was maintained throughout the procedure above 380 s. Patients were kept normothermic throughout surgery. In order to prevent major hemodynamic alterations during grafting, cardiac position was accepted only when systolic blood pressure was higher than 70 mm Hg and mixed venous oxygen saturation remained above 60%. A cell-saver suction device was used to retransfuse washed pericardial shed blood.

Microcirculatory imaging

Videomicroscopy of the sublingual microcirculation was performed with a Sidestream Dark Field (SDF) camera (Microscan Video Microscope; Microvision Medical, Amsterdam, The Netherlands). The SDF technique is a technique based on the absorbance spectrum of hemoglobin, used to visualize erythrocytes in microvessels that are localized close to the surface [15]. Sublingual microcirculatory perfusion was measured at three consecutive time points: after induction of anesthesia (Preoperative), during aortic cross-clamp time in surgery with CPB, or during distal grafting in off-pump surgery (Intraoperative), and in the first hour after admission to the Intensive Care Unit (Postoperative; Figure 1). Each measurement consisted of three video recordings of 20 seconds each at three different sublingual sites.

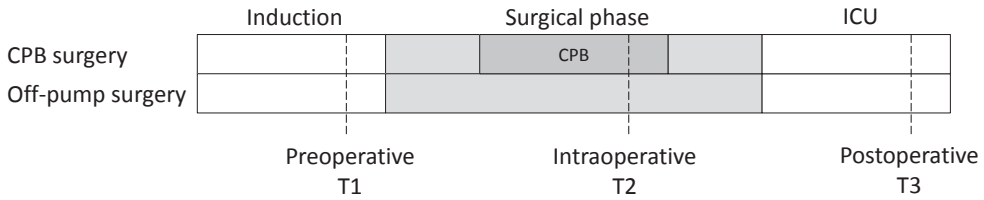

Figure 1 Schematic study protocol for both groups including the three time points at which measurements were made. The light grey area represents the surgical period, whereas the CPB duration is dark grey. CPB = cardiopulmonary bypass, ICU = Intensive Care Unit.

Microcirculatory analysis

Video clips were analyzed with automatic vascular analysis software (AVA 3.0, Microvision Medical, Amsterdam) for the microcirculatory flow profile. All analyses were performed by one of the authors, one third of the video clips was randomly reviewed by one of the other authors for conformation, both blinded for group allocation. The video screen was divided into 4 quadrants. Subsequently, quadrants were scored for the microvascular flow index (MFI), a semi-quantitative scoring scale for the quality of the microvascular flow pattern from,

ranging 0 to 3: no flow, sluggish flow, intermittent flow and continuous flow, respectively [16]. Capillary red blood cell velocities were determined in all video recordings of a random subset of 5 patients per group in order to objectify hyperdynamic microcirculatory perfusion. In each video, the RBC velocity of 10 perfused capillaries was quantified in space-time diagrams as previously described [17] or, above maximal velocity detection by space-time diagrams, the distance of frame-by-frame displacement of erythrocytes or leukocytes was determined in order to calculate the velocity [18]. For each vessel, the average of multiple velocity determinations was registered. Capillaries were selected based on their position, most centrally located capillaries were included first, as assessed by using De Backer grid line crossings [16]. Care was taken to exclude possible arterioles from velocity quantification by assessing the morphology of the microvascular bed. The fraction of hyperdynamic capillaries in quantitative analysis was assessed using a threshold at the 100th percentile of RBC velocities observed preoperatively. Additionally, the magnitude of hyperdynamic microcirculatory perfusion was determined semi quantitatively in all patients by determination of the number of quadrants per recording with predominantly hyperdynamic capillaries. Required for semi quantitative classification as hyperdynamic vessels were both 1) flashing intensity through rapid leukocyte passage and 2) difficulty to determine flow direction with the bare eye. In order to assess microcirculatory heterogeneity semi-quantitatively in all patients, the heterogeneity index was used. Heterogeneity index is calculated by dividing the range of MFI scores between quadrants by the mean MFI score, as previously used by Trzeciak et al. [19].

Data collection of hemodynamics and oxygenation parameters

Arterial and mixed venous blood gas analysis were performed at all time points, followed by thermodilution cardiac output measurement and registration of mean arterial and central venous pressure (CVP), in order to calculate the hemodynamic and oxygenation parameters as described below. During cardiopulmonary bypass, mixed venous blood was sampled from the venous reservoir instead of from the pulmonary artery catheter and pump flow was used for calculations instead of cardiac index. The following formulas were used:

C_aO_2 (arterial O_2 content; ml O_2 / dl of blood) = hemoglobin concentration (mmol/l) / 0.6206 (conversion mmol/l to g/dl hemoglobin) x 1.39 (ml O_2 / g of hemoglobin) x S_aO_2 + P_aO_2 x 0.0031;

C_vO_2 (venous O_2 content; ml O_2 / dl of blood) = hemoglobin concentration (mmol/l) / 0.6206 x 1.39 x S_vO_2 + P_vO_2 x 0.0031;

$C_{(a-v)}O_2$ (arteriovenous difference in oxygen content) = C_aO_2- C_vO_2;

DO_{2i} (systemic oxygen delivery index) = C_aO_2 x 10 (conversion ml O_2 / dl of blood to ml O_2 / l of blood) x cardiac index;

VO_{2i} (systemic oxygen consumption index) = (C_aO_2- C_vO_2) x 10 x cardiac index;

O_2ER (oxygen extraction ratio) = VO_{2i} / DO_{2i};

SVRI (systemic vascular resistance index) = (MAP – CVP) x 80 / CI.

Statistical analysis

Data were analyzed by SPSS statistical software package (version 17.0). All values are expressed as mean ± standard deviation or median with interquartile range (IQR). A Chi-square test was used to test ordinal parameters. Students T-test was used to test between groups for parametric variables. For nonparametric parameters, between-group differences at individual time points were analyzed with a Mann Whitney U test. For within-group differences, paired t-tests or Wilcoxon tests were used as appropriate. Spearman rho correlation coefficients were calculated to assess relationships between systemic variables and the number of hyperdynamic quadrants or the heterogeneity index as microcirculatory parameters. A P-value below 0.05 was considered as indicative of a statistically significant difference.

Results

Patient characteristics

Patient characteristics are presented in Table 1. No differences in preoperative parameters were observed between patients undergoing CPB or off-pump surgery, except for increased heparin and protamine administration in patients undergoing CPB.

Table 1 Patient characteristics

Description	CPB surgery (n=18)	Off-pump surgery (n=13)	P-value
Age (years)	68 ± 8	62 ± 9	0.074
Male/female (n)	15 (83%)	12 (92%)	0.245
Body surface area (m^2)	2.0 ± 0.2	2.0 ± 0.2	0.624
Median Euroscore	3 [2-5]	3 [1-4]	0.236
Platelet aggregation inhibitors (n)	18 (100%)	13 (100%)	1.000
Baseline creatinine (µmol/l)	81 ± 19	88 ± 36	0.481
Baseline hematocrit (l/l)	0.40 ± 0.03	0.42 ± 0.04	0.210
Baseline leukocyte count ($x10^9$/l)	7.6 ± 1.8	7.9 ± 1.6	0.316
Baseline thrombocyte count ($x10^9$/l)	247 ± 61	293 ± 107	0.148
Median coronary grafts (n)	4 [3-4]	4 [3-5]	0.950
Surgical duration (min)	264 ± 45	249 ± 45	0.383
CPB time (min)	113 ± 29	-	-
Total intraoperative heparin dose (mg)	509 ± 276	271 ± 82	0.006
Protamine dose (mg)	386 ± 87	257 ± 122	0.002

CPB = cardiopulmonary bypass, N = number, NS = not significant. Values are means ± SD, medians [interquartile range] or number of patients (percentage).

Hemodynamic data

Mean arterial pressure dropped approximately 15 mm Hg during surgery compared to the preoperative period in both groups. This occurred in parallel with an increase in cardiac index in the CPB group only (Figure 2). Postoperatively, mean arterial pressure remained reduced in the CPB group, while it restored in the off-pump group. In line with systemic vascular resistance, blood hematocrit declined in the CPB group with one-third after onset of extracorporeal circulation. A minor decrease in hematocrit was detected in the off-pump group. Body temperature was reduced from 36.4 ± 0.6 to 35.0 ± 0.7 °C (P<0.001) during CPB and was restored postoperatively to 36.5 ± 0.2 °C. In the off-pump group, mean preoperative temperature was 36.3 ± 0.6 °C and showed a slight increase to 36.6 ± 0.5 °C (P=0.005 vs preoperative) and 37.0 ± 0.4 °C (P<0.001 vs preoperative) intraoperative and postoperative, respectively.

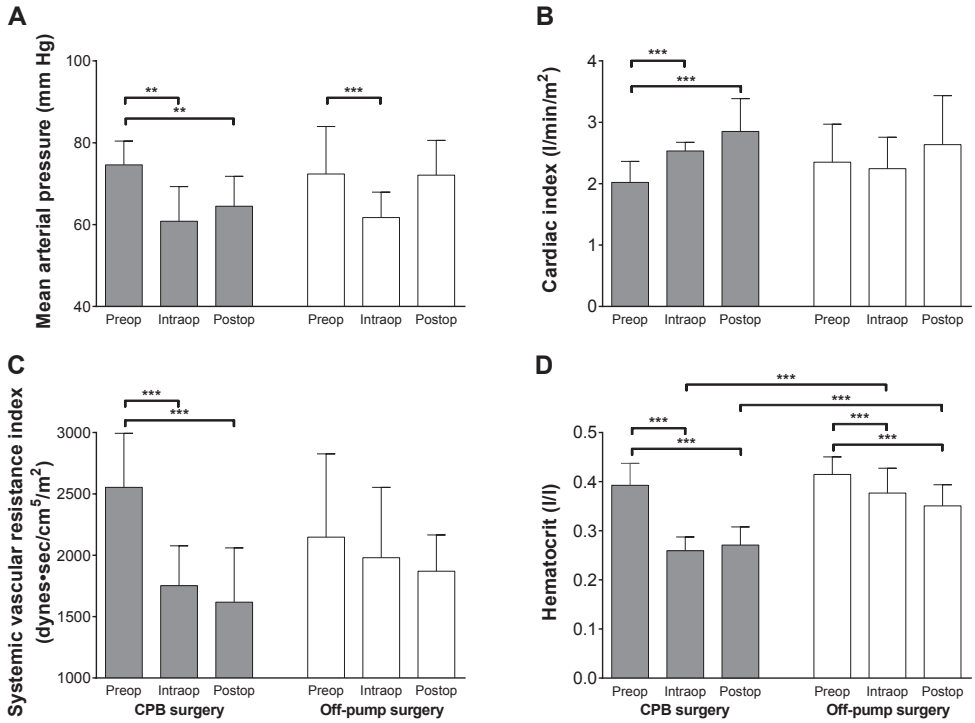

Figure 2 Systemic parameters in patients undergoing cardiac surgery with or without cardiopulmonary bypass. A, Mean arterial pressure. B, Cardiac Index. C, Systemic vascular resistance. D, Hematocrit. CPB = cardiopulmonary bypass, Intraop = Intraoperative, Preop = Preoperative, Postop = Postoperative. Data was tested with Students T-test or paired T-test for between or within group comparison, respectively. * P<0.05, ** P<0.01, *** P<0.001.

Preoperative lactate levels were at 1.1 ± 0.3 mmol/l and 1.0 ± 0.5 mmol/l (P=0.50 between groups) before on-pump and off-pump surgery, respectively. Postoperative lactate concentration of 1.6 ± 0.7 mmol/l (CPB) and 1.3 ± 0.4 mmol/l (off-pump, P=0.23 between groups) was not different between groups. No difference in administration of dopamine, nitroglycerine or phenylephrine was observed between groups (Table 2).

Table 2 Vasoactive medication

		CPB Surgery	Off-pump surgery	P value
Dopamine n (%)	**Preoperative**	6 (33%)	7 (54%)	NS
	Intraoperative	5 (28%)	7 (54%)	NS
	Postoperative	13 (72%)	7 (54%)	NS
Nitroglycerine n (%)	**Preoperative**	6 (33%)	3 (23%)	NS
	Intraoperative	7 (39%)	3 (23%)	NS
	Postoperative	12 (66%)	4 (31%)	NS
Phenylephrine n (%)	**Preoperative**	6 (33%)	4 (31%)	NS
	Intraoperative	4 (22%)	2 (15%)	NS
	Postoperative	2 (11%)	2 (15%)	NS

CPB= cardiopulmonary bypass, N = number, NS = not significant. Data was tested with Chi square test. Values are number of patients (percentage).

Microcirculatory flow profile

The median preoperative microvascular flow index was 3.0 (2.6-3.0) and declined to 2.7 (2.4-3.0; P=0.069; intraoperative) and 2.5 (2.0-2.9; P=0.049, r=0.46; postoperative) in the CPB group. There were no alterations in MFI during off-pump surgery observed, with median MFI at 2.9 (2.8-3.0), 2.9 (2.8-3.0; P=0.674 vs preoperative) and 2.9 (2.8-3.0; P=0.352 vs preoperative), respectively. The semi-quantitative indices of microcirculatory heterogeneity (Figure 3A) and hyperdynamic microcirculatory perfusion (Figure 3B) increased substantially after onset of CPB. There were no alterations in the microcirculatory flow profile observed in the group undergoing off-pump procedures (Figure 3). The microcirculatory flow profile remained disturbed after weaning from extracorporeal circulation in the CPB group. This occurred in parallel with an augmented cardiac index and reduced levels of hematocrit, systemic vascular resistance and mean arterial pressure. Microcirculatory perfusion remained stable in the off-pump group throughout the perioperative period.

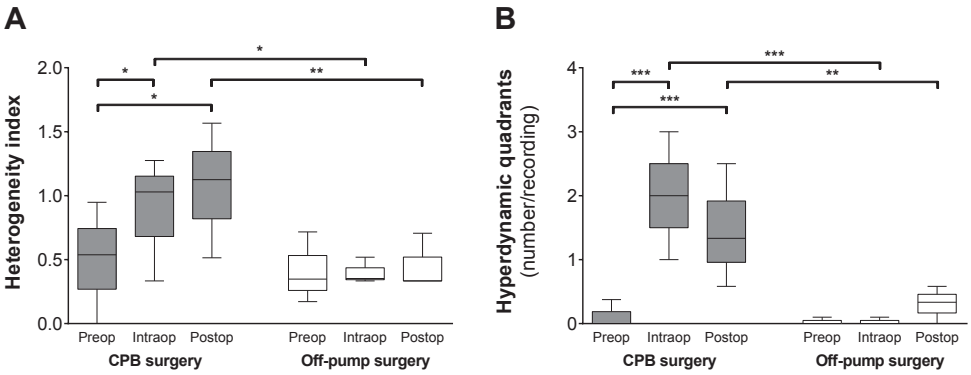

Figure 3 Sublingual microcirculatory parameters of heterogeneity and hyperdynamic perfusion. A, Heterogeneity index. B, Hyperdynamic microcirculatory perfusion. CPB = cardiopulmonary bypass, Intraop = Intraoperative, Preop = Preoperative, Postop = Postoperative. Data was tested with Mann Whitney U test or Wilcoxon test for between or within group comparison, respectively. * P < 0.05, ** P < 0.01, *** P<0.001.

Quantification of capillary red blood cell velocity

Preoperative capillary red blood cell velocity analysis revealed no difference between the CPB group (369 [191-678 µm/s]) and the off-pump group (413 [220-750 µm/s]; P=0.56). CPB was associated with increased median velocities as compared to the off-pump procedure (1600 [899-2500 µm/s] versus 380 [190-480 µm/s]; P< 0.001), and this persisted during the postoperative measurement (Figure 4).

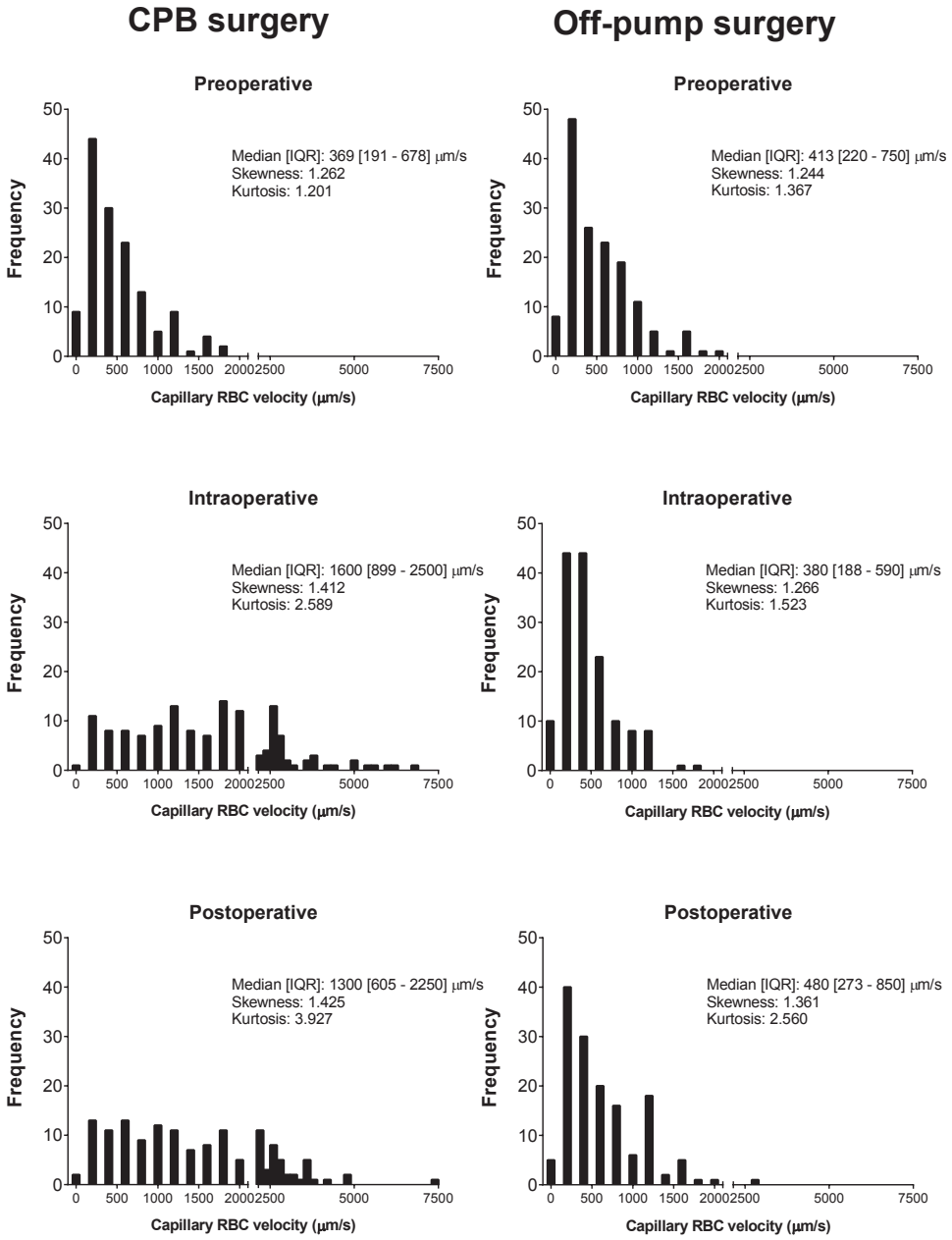

Figure 4 Histogram of capillary red blood cell velocity, pooled for patients undergoing cardiac surgery with (n=5) or without (n=5) cardiopulmonary bypass. CPB = cardiopulmonary bypass, RBC = Red blood cell.

The 100th percentile of all preoperative capillary RBC velocities was 2000 µm/s. The percentage of capillaries that were hyperdynamic (defined as higher RBC velocity than P100 preoperatively) increased during CPB to 31% of all capillaries (maximal RBC velocity 6750 µm/s) and after CPB to 29% of all capillaries (maximal RBC velocity 7350 µm/s). In the off-pump group, no hyperdynamic capillaries were observed (0% intraoperatively and 1% postoperatively; P<0.001 between groups for both time points).

Oxygenation parameters

In parallel with the development of increased microcirculatory heterogeneity and hyperdynamic capillary perfusion, the arteriovenous difference in blood oxygen content diminished after onset of CPB by 50%, and remained reduced postoperatively (Table 3). Moreover, mixed venous O_2 saturation increased, even though acute hemodilution caused a decline in oxygen delivery. Systemic oxygen consumption declined from 72 ± 22 to 47 ± 14 ml/min/m^2 (P=0.002) during CPB, along with reduced oxygen delivery, although the oxygen extraction ratio was low. After weaning from CPB, a recovery of oxygen consumption and delivery was observed, although $C_{(a-v)}O_2$ remained diminished. In contrast, oxygenation parameters remained largely unaltered in the off-pump group.

Table 3 Oxygenation parameters

		CPB Surgery	Off-pump surgery	P value
S_vO_2 (%)	**Preoperative**	78 ± 7	80 ± 8	0.593
	Intraoperative	83 ± 4†	75 ± 5	0.000
	Postoperative	75 ± 10	70 ± 9†	0.148
$C_{(a-v)}O_2$ (ml/dl)	**Preoperative**	3.6 ± 1.1	3.6 ± 1.4	0.998
	Intraoperative	1.8 ± 0.6†	4.1 ± 1.1	0.000
	Postoperative	2.7 ± 1.0†	4.5 ± 1.6	0.001
DO_{2i} (ml/min/m^2)	**Preoperative**	347 ± 77	412 ± 113	0.119
	Intraoperative	281 ± 32†	409 ± 120	0.000
	Postoperative	318 ± 98	504 ± 101	0.000
VO_{2i} (ml/min/m^2)	**Preoperative**	72 ± 22	89 ± 32	0.152
	Intraoperative	47 ± 14†	102 ± 22	0.000
	Postoperative	71 ± 23	117 ± 20	0.000
O_2ER (%)	**Preoperative**	22 ± 7	20 ± 6	0.680
	Intraoperative	17 ± 5†	24 ± 4	0.001
	Postoperative	24 ± 9	24 ± 3	0.926

$C_{(a-v)}O_2$ = Arteriovenous oxygen content difference, CPB = cardiopulmonary bypass, DO_{2i} = Oxygen delivery, O_2ER = Oxygen extraction ratio, SvO_2 = mixed venous oxygen saturation, VO_{2i} = Oxygen consumption. Values are means ± SD. †P<0.05 vs Preoperative.

Correlation of systemic variables with microcirculatory parameters

Spearman coefficients were calculated to assess associations between systemic variables and hyperdynamic microcirculatory perfusion or the heterogeneity index. Inverse, but weak correlations of hyperdynamic microcirculatory perfusion with $C_{(a-v)}O_2$ (Rho = -0.33, P<0.001) and VO_{2i} (Rho=-0.27, P=0.008) were detected. Moreover, a reduction in hematocrit was associated with a moderate increase in hyperdynamic quadrants (Spearman's Rho = -0.51, P<0.001) and weak alteration in the heterogeneity index score (Rho = -0.28, P=0.001). No correlations with other hemodynamic or blood gas derived variables or administered vasoactive medication were observed.

Postoperative outcome

ICU stay was 1 [1-1] and 1 [1-1] days in on-pump and off-pump groups, respectively (P=0.798). Two patients in the CPB group developed postoperative acute kidney injury (AKI), one of which died three weeks postoperatively, whereas the other patient regained pre-existent renal function. No acute kidney injury or mortality was observed in the off-pump group (P=0.497 for acute kidney injury, P=1.000 for mortality between groups). Both patients developing acute kidney injury showed increased intraoperative (+0.63 and +0.56) and postoperative heterogeneity index (+1.38 and +0.59) and decreased postoperative MFI scores (-1.04 and-0.47) as compared to preoperative values. New onset postoperative atrial fibrillation developed in 2 patients undergoing on-pump surgery and in 1 patient undergoing off-pump surgery (P=1.000 between groups). No myocardial or cerebral infarction occurred in the current study population.

Discussion

This study is the first to show that the onset of cardiopulmonary bypass induces acute microcirculatory flow alterations, with increased microcirculatory heterogeneity and a large fraction of hyperdynamically perfused capillaries, both persisting in the early postoperative phase. Quantification of capillary blood velocity during CPB revealed high flow rates, associated with a reduction in systemic oxygen extraction. These observations were absent in patients undergoing off-pump cardiac surgery.

Normal capillary red blood cell velocities may differ considerably within and between tissues, but are generally thought to range between 100 and 800 µm/s, with a maximum of 1600 µm/s [5,20-22]. The threshold we currently applied for hyperdynamic capillaries was based on the maximum RBC velocity observed preoperatively, at 2000 µm/s well above normal levels. During and after cardiac surgery with CPB, almost one-third of the capillaries are perfused at very high velocities, while we found no hyperdynamic capillaries in off-pump surgery. To our best knowledge, capillary blood velocities up to 7350 µm/s have not been reported in literature. It is conceivable that the concomitant shear stresses may inflict damage to the capillary endothelium or the endothelial glycocalyx and subsequently contribute to obstructed capillaries [23,24].

High capillary blood velocities limit oxygen exchange and thus cause systemic arteriovenous shunting of oxygenated hemoglobin [12]. Despite a marked hemodilution and a reduced microvascular flow index, $C_{(a-v)}O_2$ diminished by 50% during CPB in the current study. The reduction in $C_{(a-v)}O_2$ occurred both intra- and postoperatively in parallel with an increased number of capillaries demonstrating flow velocities that cannot support adequate RBC oxygen offloading. Additionally, an inverse correlation between $C_{(a-v)}O_2$ and hyperdynamic capillaries was detected. In line with the current results, a previous investigation by our group showed that the onset of extracorporeal circulation led to an immediate increase in microvascular hemoglobin saturation, reflecting reduced microcirculatory oxygen extraction [17]. Our current observations therefore support the existence of microvascular shunting through high velocity capillaries in a patient population undergoing cardiopulmonary bypass. A microvascular shunting phenomenon as observed in the current investigation, has often been hypothesized to exist during critical illness [25-28]. However, to date no such phenomenon has been visualized in humans [29]. The presence of hyperdynamic capillaries during septic shock was recently not detected in patients with septic shock [29]. Macrocirculatory variables were however not hyperdynamic in that investigation, which might have precluded the observation of hyperdynamic microcirculatory perfusion [29]. An alternative explanation for the lack of detection of microvascular shunting in the study of Edul et al. may be the prolonged time between onset of disease and microcirculatory observation for patients with sepsis [30,31]. We have demonstrated in the current study that microvascular shunting

occurs within an hour after onset of an injurious stimulus, similar to experimental studies that have observed hyperdynamic capillaries [5,6,32]. It may therefore be conceivable that microvascular shunting is an early phenomenon that may be more difficult to detect with progression of microcirculatory disturbances.

The increase in microcirculatory heterogeneity induced by CPB may lead to impairment of tissue oxygen extraction [3,5]. In the current study, systemic oxygen consumption declined by 35% during cardiopulmonary bypass in the current study, which could not be explained by mild hypothermia alone [33]. Although the exact cause of this large reduction in VO_2 remains unknown from the present investigation and a contribution of mitochondrial dysfunction cannot be excluded [34], it is likely that VO_2 was limited by microcirculatory heterogeneity due to local mismatching of oxygen supply and demand [3,7]. Low oxygen consumption is associated with an increase in anaerobic CO_2 production and increased lactate levels [35]. Hyperlactatemia has been associated with increased postoperative morbidity and mortality following cardiac surgery [36], although this was not observed in the current population with low Euroscores. More complex cardiac surgery with longer CPB duration is associated with increased postoperative morbidity and mortality and patients undergoing these procedures are thus likely to have more severe microcirculatory impairment. The work of Trzeciak et al. supports the association between microcirculatory failure and poor outcome, since the heterogeneity index in the microcirculation early in the course of septic shock was predictive of mortality [11]. The relationship between microcirculatory disturbances and poor clinical outcome was confirmed in multiple several patient populations [8-10], but this has not yet been established for cardiac surgery. We currently did observe deterioration of microvascular perfusion parameters in the two patients developing AKI, but the current study was not designed to detect a relationship with clinical outcome.

Our observation may be an important factor in explaining several clinical observations on oxygenation parameters, as the difference between microvascular and venous PO_2 values observed in critical disease states [37,38]. Moreover, we demonstrate that microcirculatory alterations can hinder the clinical interpretation of S_vO_2, as previously it has been demonstrated that venous hyperoxia after cardiopulmonary resuscitation is associated with poor outcome [39]. This is supported by recent reports on ICU patients by Velissaris et al., in which high S_vO_2 levels were not indicative of adequately optimized circulatory management [40], and by Monnet and coworkers, showing that S_vO_2 could not predict increased oxygen consumption after an intravascular volume expansion in fluid responders [41]. Most illustrative is the recent study by Pope and colleagues in patients admitted to the emergency department, showing that high central venous oxygen saturation is associated with increased mortality, hypothetically through microcirculatory failure [27]. Finally, a possibly erroneous interpretation of S_vO_2 values should be taken into account when using it as a red blood cell transfusion trigger for cardiosurgical patients [42].

Although several factors might theoretically be responsible for the alterations of the microcirculatory flow profile during CPB, the current study does not differentiate between the different causes. A one-third reduction in systemic vascular resistance was observed after onset of CPB. During arteriolar vasodilation, a reduction in blood pressure drop over the resistance vessels leads to a higher pressure difference over the capillary bed [19], which may theoretically increase the number of hyperdynamically perfused capillaries. However, the relationship between macrocirculatory parameters and microcirculatory perfusion has been found to be loose in multiple investigations [43], and the contribution of a reduction in systemic vascular resistance to microvascular perfusion patterns may therefore be questionable. Increased microcirculatory heterogeneity is hypothesized to be a sign of impaired vascular reactivity [5], a phenomenon that is known to occur during CPB [44] and may be aggravated by nonpulsatile flow as is present during CPB [45]. Previously, we have found that pulsatile flow during CPB is associated with improved recovery of perfused microvascular density [1], but the effect of pulsatility on vasoreactivity or microcirculatory heterogeneity is still unknown. Finally, acute hemodilution during onset of cardiopulmonary bypass contributes to reduced resistance to blood flow due its decrease in blood viscosity. We found that reduced hematocrit was correlated to both increased heterogeneity and hyperdynamic capillary perfusion in the current study. In experimental observations, however, hemodilution alone caused a maximum of 40% increase in arteriolar blood velocity [46], and capillary velocity never exceeded 2000 µm/s at hematocrit levels down to 0.20 l/l. [47]. Miniaturized extracorporeal circulation systems cause less hemodilution than conventional systems, but the associated reduction in hemodilution led to only a minor improvement in microcirculatory perfusion [48]. Hemodilution as observed in the current population may therefore contribute to microcirculatory disturbances, but only to a minor extent.

The current study is limited by the highly regional microcirculatory measurement method. Although it has been challenged [49], most studies have found that the sublingual microvascular perfusion is closely correlated to the visceral microvasculature [50-52]. Moreover, all microvascular beds are exposed to the same hemodynamic alterations during the study period, and the disturbances in whole body oxygenation parameters suggest that the current observations are a systemic phenomenon.

In conclusion, the current study provides the first direct human evidence for a systemic microvascular shunting phenomenon following acute physiological disturbances, as occurs after onset of cardiopulmonary bypass but not during off-pump surgery. A hypothesis of impaired oxygen extraction caused by hyperdynamic capillaries was supported by reduced blood arteriovenous oxygen difference and low systemic oxygen extraction. This observation increases the understanding of acute microcirculatory pathophysiology, which is necessary before interventions to preserve microcirculatory perfusion can be examined. The mechanism by which these microcirculatory alterations develop are still to be investigated. Additionally, it

remains to be elucidated whether an acute induction of microcirculatory heterogeneity and arteriovenous shunting by cardiopulmonary bypass is associated with increased morbidity or mortality.

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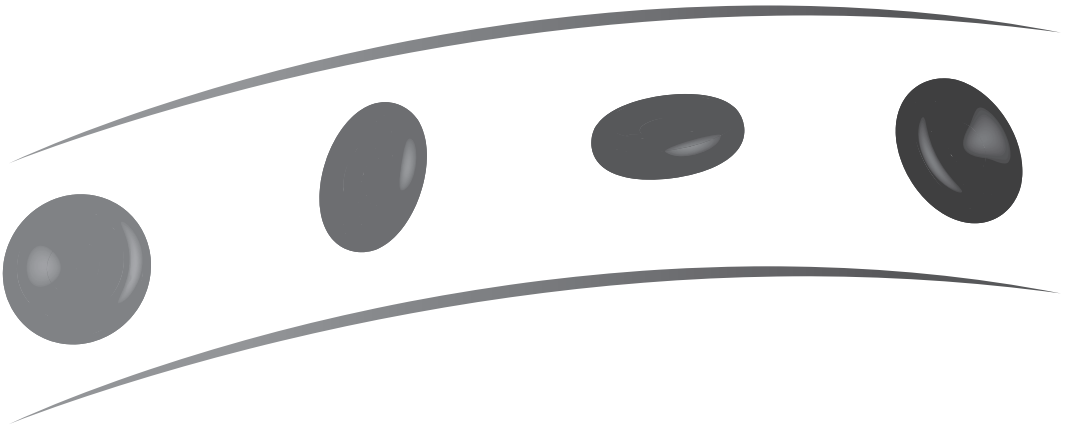

CHAPTER 6

Endothelial hyperpermeability after cardiac surgery with cardiopulmonary bypass as assessed using an in vitro bioassay for endothelial function

N.J. Koning, M.A.H. Overmars, C.E. van den Brom, J. van Bezu, L.E. Simon, A.B.A. Vonk, A.R.J. Girbes, G.P. van Nieuw Amerongen, C. Boer.

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Abstract

Background: The mechanisms behind increased endothelial permeability following use of cardiopulmonary bypass (CPB) have not been elucidated. Using a bioassay for endothelial barrier function we investigated whether the cause of endothelial hyperpermeability should be sought in plasmatic alterations, and can be modulated by application of pulsatile flow during CPB as protective intervention for the endothelium.

Methods: Plasma samples of patients undergoing cardiac surgery with nonpulsatile (n=20) or pulsatile flow CPB (n=20) were obtained before (pre-CPB) and after CPB (post-CPB), and upon intensive care unit (ICU) arrival. Changes in plasma endothelial activation and adhesion markers were determined by ELISA. Using Electric Cell-substrate Impedance Sensing (ECIS) of human umbilical vein endothelial monolayers, the effects of plasma exposure on endothelial barrier function were assessed and expressed as resistance.

Results: CPB was associated with increased P-selectin, Vascular Cell Adhesion Molecule and von Willebrand Factor plasma levels and the Angiopoietin-2 to Angiopoietin-1 ratio, irrespective of the flow profile. After CPB, plasma samples induced loss of endothelial resistance of 21% and 23% in nonpulsatile and pulsatile flow groups, respectively. The negative effect of plasma exposure of endothelial cells on barrier function was still present upon intensive care admission (ICU). The reduction in endothelial resistance after exposure to post-CPB plasma could not be explained by CPB-induced hemodilution.

Conclusion: The change in the plasma fingerprint during CPB is associated with impairment of in vitro endothelial barrier function, which occurs irrespective of the application of a protective pulsatile flow profile during CPB.

Introduction

Patients undergoing cardiac surgery with cardiopulmonary bypass (CPB) are at risk to develop tissue oedema due to inflammatory processes, fluid overload and augmented fluid extravasation [1,2]. Postoperative oedema may contribute to the development of complications, in particular acute lung injury [3].

Fluid extravasation during cardiac surgery is mainly attributed to altered gradients in the hydrostatic and oncotic pressure due to crystalloid and colloid fluid administration, loss of glycocalyx and basement membrane integrity and endothelial barrier dysfunction [4-6]. The etiology of endothelial hyperpermeability during cardiac surgery is however not well understood. Previous studies suggested that endothelial barrier function during (CPB) is attenuated by volatile anesthesia [7], a nonpulsatile blood flow profile [8,9], the release of pro-inflammatory mediators [10,11], endothelial activation [11-13], hemodilution [14], the use of heparin [15] and hypothermia [16]. In porcine studies, cardiopulmonary bypass was associated with degradation of endothelial adherens junctions in tissue sections [17], reduced myocardial vascular VE-cadherin, beta-catenin and gamma-catenin levels [18], and fluid extravasation [7,19,20]. This could not be diminished with anti-inflammatory agents [16] or pulsatile blood flow [19].

Most aforementioned findings were obtained in animal studies [7,14,16-20] or limited to the evaluation of plasma markers [8,10,11] or immunohistochemistry in arterial biopsies [12]. Moreover, the data are inconclusive whether loss of endothelial barrier function during CPB is dependent on the blood flow profile [21], or specifically mediated by alterations in the plasma footprint of biomarkers. Using a bioassay for endothelial barrier function we here investigated the effect of plasma obtained from patients undergoing cardiac surgery with nonpulsatile CPB on endothelial cell permeability. Furthermore, we evaluated whether the application of a pulsatile blood flow profile during CPB, leading to higher peak shear stress levels which are associated with improved endothelial barrier function in vitro [22], and which we previously used as vascular function-preserving intervention [23], could alter the effects of human plasma on endothelial permeability.

Methods

Study design

This single center, randomized trial (NTR2940) was approved by the Human Subjects Committee of the VU University Medical Center (NL34947.029.10) and all participants signed informed consent. Forty patients aging 50-85 years undergoing elective coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass were included. Exclusion criteria were an emergency operation or reoperation, insulin-dependent diabetes mellitus or anemia (hemoglobin <5.5 mmol/l). Patients were assigned to the nonpulsatile flow (n=20) or pulsatile flow group (n=20) by randomization using envelope drawing. The study flow diagram is shown as Figure 1.

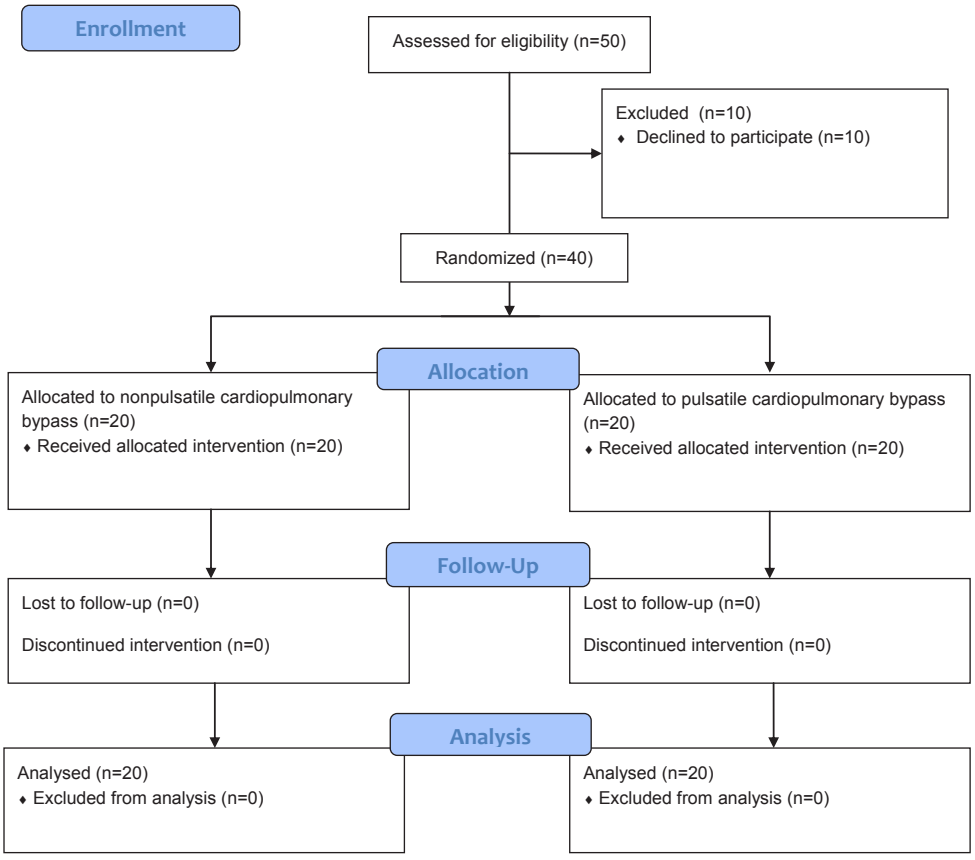

Figure 1 Flow diagram of the trial.

Anesthesia and surgery

A standard anesthesia protocol was applied in all patients as described before [23]. Patients received dexamethasone (1 mg/kg), cefazolin (1000 mg) and tranexamic acid (2 g) according to local protocols. Heparin (300 IU/kg) was used to achieve an activated clotting time of >480 s. The extracorporeal circuit consisting of a centrifugal pump head (Sorin, Mirandola, Italy), a polyvinyl heparin coated tubing system and a hollow fibre oxygenator oxygenator (Affinity, Medtronic, Minneapolis, USA). The system was primed with 1 liter of Gelofusin® (Braun, Melsungen, Germany), 250 ml lactated Ringer's solution (Baxter BV, Utrecht, Netherlands) containing cefalozin (1000 mg; Eli Lilly Nederland BV, Nieuwegein, Netherlands), mannitol (100 ml 20%; Baxter BV), sodium bicarbonate (50 ml 8.4%; Braun Melsunger AG) and porcine heparin (5000 IU). CPB blood flow was maintained at 2.2-3.0 l/min/m² with mild hypothermia (34°C). Heparin was neutralised by protamine. A cell saver (Autolog, Medtronic, Minneapolis, USA) was routinely used for autologous red blood cell concentration. During aortic cross-clamping, the flow character of the centrifugal pump was set to nonpulsatile in the nonpulsatile flow group or pulsatile in the pulsatile flow group (60 accelerations per minute) according to a standard cardiopulmonary bypass protocol [23]. Area under curve (AUC) analysis was performed on the arterial blood pressure waveform before CPB and during aortic cross-clamping in order to quantify the pulsatility delivered by the centrifugal pump.

Blood sampling

Blood samples were collected before anesthesia induction (pre-CPB) and three minutes (post-CPB) and 60 minutes (ICU) following protamine infusion, and immediately centrifuged at 4000 rpm during 10 minutes at 4°C. The plasma supernatant was centrifuged for 5 minutes at 12000 rpm to achieve platelet free plasma (PFP). PFP was snap frozen in liquid nitro gen and stored at-80°C.

Plasma analysis

Plasma samples were analysed for P-selectin, ICAM-1, VCAM-1, von Willebrand Factor (vWF), Vascular Endothelium Growth Factor (VEGF; all from AbCam, Cambridge, UK), Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2) and soluble Tie2 receptor (sTie2; R&D Systems, Minneapolis, USA) levels using commercially available enzyme-linked immunosorbent assays. Additionally, Post-CPB and ICU values were corrected for dilution due to cardiopulmonary bypass as reflected by the relative decrease in hematocrit immediately after onset of CPB as compared to pre-CPB hematocrit values.

HUVECs

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated and cultured as described before [24]. HUVECs were isolated from human umbilical cords and subsequently cultured on gelatine-coated well plates in M199 medium at 37°C, 5% CO_2 and 95% air atmosphere.

Electric Cell-substrate Impedance Sensing

Electric Cell-substrate Impedance Sensing (ECIS) is a technique to measure impedance of endothelial cells (Applied BioPhysics, Troy, USA), as previously described [24,25]. Using a 96-wells ECIS array (0.3 cm^2 per well containing 20 gold-electrodes of 350 µm), a confluent monolayer of endothelial cells was cultured in each well, and impedance, resistance and capacity were measured.

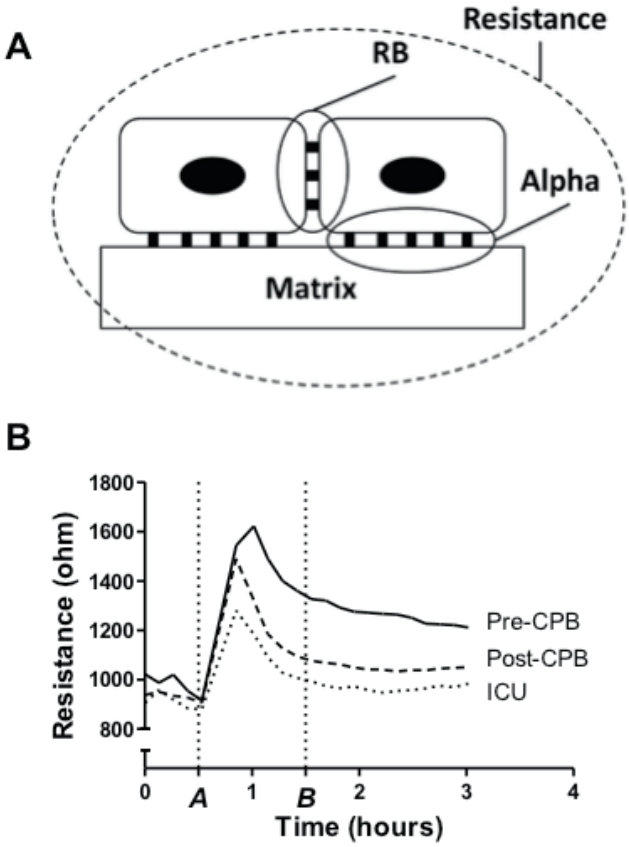

Figure 2 Electric Cell-substrate Impedance Sensing. Schematic representation of Electric Cell-substrate Impedance Sensing (ECIS; panel A). Two endothelial cells are connected by cell-cell junctions and attached to the extracellular matrix by cell-matric connections. ECIS measurements provide overall information on endothelial barrier function (resistance), which can be separated into a component for cell-cell binding (Rb) and cell-matrix binding (alpha). Increases in resistance, Rb and alpha indicate enhanced endothelial barrier function. Panel B represents a typical example of the overall resistance during exposure of endothelial cells to plasma derived from a patient at baseline (pre-CPB; cardiopulmonary bypass), after CPB (post-CPB) and upon intensive care unit (ICU) admission. Following a 30-minute exposure of the endothelial cells to 1% HSA, plasma is added which induces a transient rise in resistance (point A), followed by a steady state (point B). The steady state values were further used to define the effect of cardiopulmonary bypass on endothelial barrier function in nonpulsatile and pulsatile patient groups.

ECIS software (v1.2.65.0 PC, Applied BioPhysics, Troy, USA) was used to calculate the level of overall resistance, cell-cell integrity (Rb) and cell-matrix integrity (alpha) [26]. Figure 2 shows a schematic overview of the overall resistance, Rb and alpha parameters that can be retrieved from ECIS measurements (panel A). Panel B shows a typical example of the effects of plasma obtained from one patient before cardiopulmonary bypass (pre-CPB), after bypass (post-CPB) and upon ICU admission on cellular resistance.
The values for the overall resistance, Rb and alpha at 60 minutes following plasma infusion were used for further statistical evaluation of our data. All measurements were performed in duplicate.

ECIS materials

For HUVEC experiments the following materials were used: Bare medium (bM199) consisting of Medium 199 supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Biowhittaker, Verviers, Belgium); Complete medium (cM199) consisting of bare medium (bM199) supplemented with 10% heat-inactivate new-born calf serum (Gibco, Grand Island, NY, USA), 10% human serum (from a local blood bank, pooled serum of 10-20 healthy donors, stored at 4°C), 20 mmol/l HEPES (pH 7.3), 2 mmol/l glutamine (both from Biowhittaker), 5 U/ml heparin (Leo Pharmaceutical Products, Weesp, The Netherlands), 150 µg/ml crude endothelial cell growth factor prepared from bovine hypothalamus; 1% HSA solution (dilution of human serum albumin in bM199; Sanquin CLB, Amsterdam, The Netherlands); Trypsine (Gibco); tissue culture plastics (Costar; Cambridge, MA, USA) and ECIS culture plates with a Z-Theta measurement device and analysis software (Applied BioPhysics, Troy USA).

ECIS study protocol

Confluent passage one HUVECS were transferred to a gelatine-coated 96-wells ECIS culture plate. After 72 hours of culturing in cM199 medium, the ECIS device was used for continuous, multi-frequency scanning to confirm a confluent monolayer. Following initiation of ECIS measurements, confluent monolayers were subsequently exposed to 1% HSA for 90 minutes followed by the addition of 10% platelet free plasma (PFP). ECIS measurements were then continued for 4 hours.

Effects of hemodilution

In order to investigate whether the level of hemodilution a separate experiment was performed using multiple concentrations of plasma samples obtained before or after cardiopulmonary bypass. Two gelatine 8-wells ECIS culture plates were seeded with passage I HUVECs, followed by a culture period of 72 hours. After confirmation of a confluent monolayer, cM199 medium was replaced by 1% HSA medium. Pre-CPB and post-CPB plasma samples of one patient were mixed with 1% HSA medium in different ratios of human plasma and medium: 3:10 (30%

plasma; 3.3x diluted), 2:10 (20% plasma; 5x diluted) and 1:10 (10% plasma; 10x diluted) for post-CPB samples and 2:10 (20% plasma; 5x diluted) and 1:10 (10% plasma; 10x diluted) for pre-CPB samples. It was hypothesized that 5x dilution of pre-CPB samples should have a comparable effect on endothelial barrier function to 3x dilution of post-CPB samples in case of a prominent role of hemodilution. A 1% HSA solution without plasma served as negative control. ECIS measurements were performed in duplicate as described above.

Statistical analysis

Data were analysed using SPSS (version 19.0; IBM, New York, USA). Data were expressed as mean (standard deviation) or frequencies. Repeated measurement ANOVA (RM) was used to test differences over time in endothelial barrier function and vascular mediators between the nonpulsatile and pulsatile flow CPB group. Paired t-tests or Mann-Whitney U tests were used to compare time points within one group, as appropriate. Correlations between endothelial barrier function parameters and patient characteristics or postoperative outcome parameters were retrieved with Pearson correlation tests. A P-value <0.05 was considered as indicator for a statistical significant difference.

Results

General patient characteristics

The study included patients undergoing cardiopulmonary bypass (CPB) with nonpulsatile flow (n=20) or pulsatile flow (n=20). Typical examples of radial arterial blood pressure waveforms during nonpulsatile (panel A) and pulsatile cardiopulmonary bypass (panel B) are depicted in Figure 3.

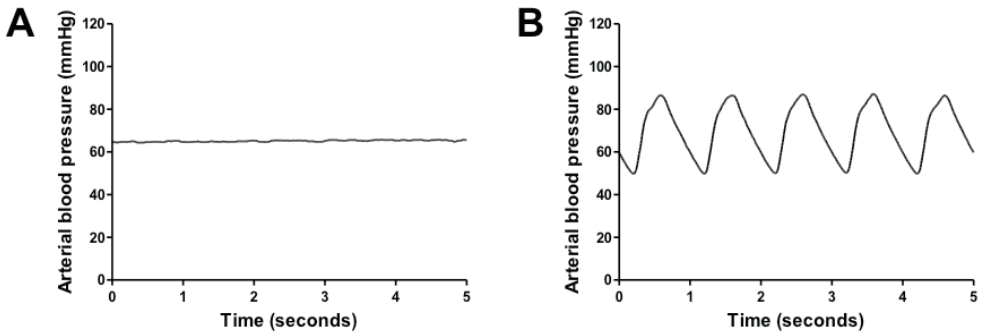

Figure 3 Nonpulsatile and pulsatile arterial blood pressure waveform. Typical examples of radial arterial blood pressure waveforms during cardiopulmonary bypass with nonpulsatile flow (panel A) and pulsatile flow (panel B).

In the pulsatile group, the AUC of the arterial pulse on the blood pressure waveform during aortic cross-clamping was 70% (23) of baseline (n=6), whereas this was 0% (0) of baseline (n=6) in the nonpulsatile group. There were no differences in baseline patient characteristics between groups (Table 1).

Table 1 Patient characteristics

Description	Nonpulsatile flow	Pulsatile flow
N	20	20
Age (years; median (IQR))	66 (64-74)	70 (65-72)
Males (n)	16 (80%)	18 (90%)
BMI (kg/m²)	27.5 (3.4)	28.7 (3.0)
BSA (m²)	2.0 (1.7)	2.1 (1.9)
Euroscore (median (IQR))	3 (2-6)	4 (2-5)
CPB time (min)	108 (24)	105 (23)
Aortic cross-clamp time (min)	72 (20)	70 (21)
Anastomoses (median (IQR))	4 (3-5)	4 (3-5)
Lowest temperature during CPB (°C)	35.1 (0.6)	35.1 (0.3)
Hematocrit (l/l)		
Pre-CPB	0.39 (0.04)	0.40 (0.04)
Post-CPB	0.27 (0.04)	0.30 (0.03)
ICU	0.33 (0.04)	0.33 (0.04)

Values are displayed as means (standard deviations) or medians (interquartile range). BMI = Body mass index, BSA = Body surface area, CPB = cardiopulmonary bypass, IQR = interquartile range. There were no statistical differences among groups.

Mediators of inflammation and endothelial activation

To study whether nonpulsatile and pulsatile cardiopulmonary bypass were differently associated with a pro-inflammatory response and endothelial activation, we evaluated alterations in mediators of inflammation and endothelial activation in human plasma derived before and after cardiopulmonary bypass (Figure 4). Cardiopulmonary bypass was associated with an increase in P-selectin (panel A), while ICAM-1 levels remained unaltered (panel B). VCAM-1 (panel C) and vWF (panel D) transiently increased following cardiopulmonary bypass, but restored towards baseline levels upon ICU admission. VEGF levels strongly decreased following cardiopulmonary bypass, and were only slightly restored upon ICU admission (panel E). There were no differences in the changes of mediators of inflammation and endothelial activation between patients subjected to nonpulsatile or pulsatile blood flow profiles during cardiopulmonary bypass.

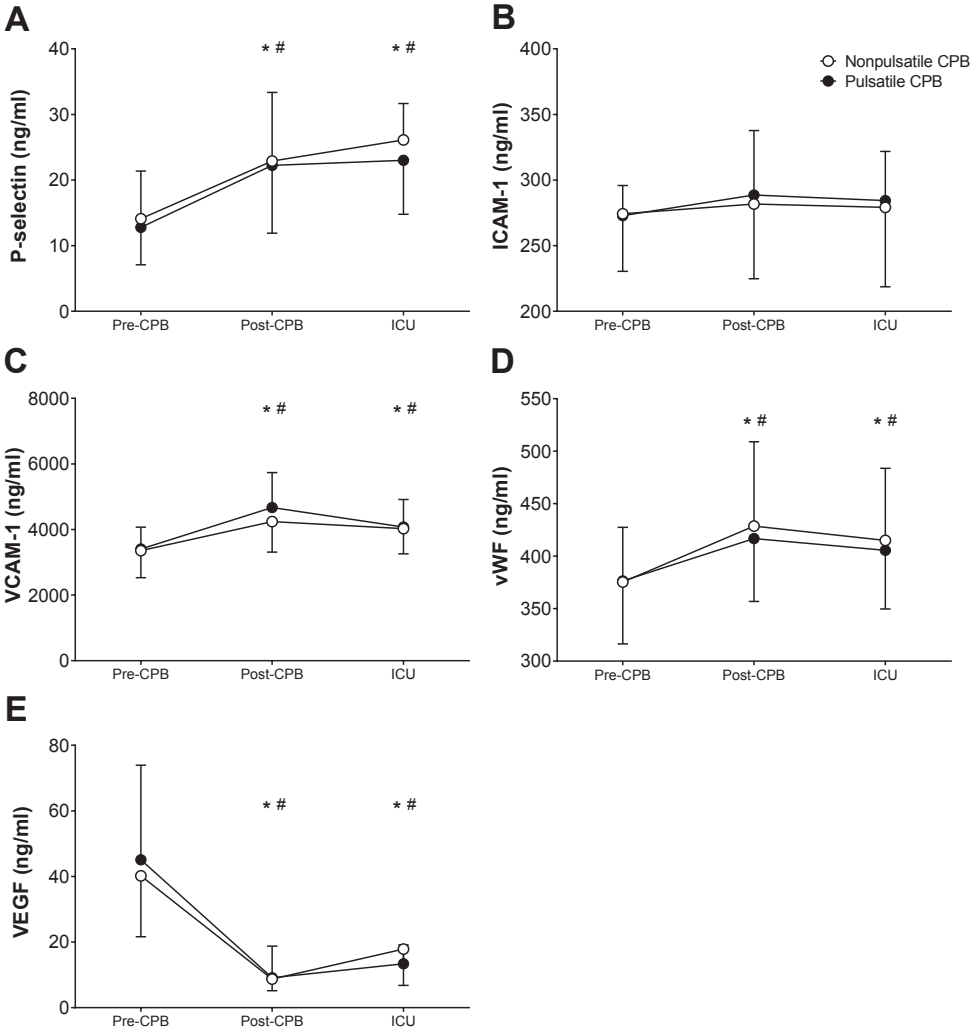

Figure 4 Plasma levels of vascular mediators. Changes in the vascular mediators P-selectin (panel A), Intercellular Cell Adhesion Molecule (ICAM-1; panel B), Vascular Cell Adhesion Molecule (VCAM-1; panel C), von Willebrand Factor (vWF; panel D) and Vascular Endothelial Growth Factor (VEGF; panel E) in patients before cardiopulmonary bypass (pre-CPB), after cardiopulmonary bypass (post-CPB) and upon intensive care unit (ICU) admission. # P<0.001 vs pre-CPB values in nonpulsatile flow group; * P<0.001 vs pre-CPB values in pulsatile flow group.

Figure 5 shows the alterations in plasma levels of Tie2, Ang-1 and Ang-2. Both nonpulsatile and pulsatile flow were associated with postoperative reductions in soluble Tie2 and Ang-1 levels, whereas the pro-endothelial barrier dysfunction molecules Ang-2 increased following CPB. The Ang-2/Ang-1 ratio increased threefold in both groups in blood sampled during ICU admission as compared to baseline values. There were no differences in Tie2, Ang-1 or Ang-2 levels between pulsatile or nonpulsatile groups.

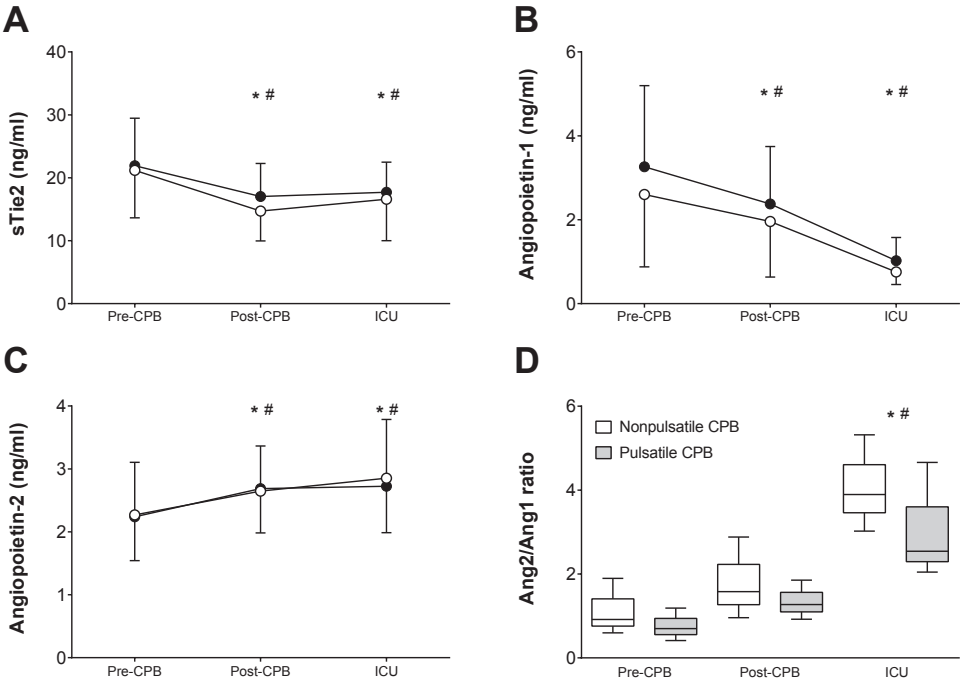

Figure 5 Plasma levels of soluble Tie2 and Angiopoietin 1 and 2. Changes in soluble Tie2 (panel A), Angiopoietin-1 (Ang-1; panel B), Angiopoietin-2 (Ang-2; panel C) and the Ang-2/Ang-1 ratio (panel D) in patients before cardiopulmonary bypass (pre-CPB), after cardiopulmonary bypass (post-CPB) and upon intensive care unit (ICU) admission. # P<0.001 vs pre-CPB values in nonpulsatile flow group; * P<0.001 vs pre-CPB values in pulsatile flow group.

Endothelial barrier function

The effect of human plasma derived before and after nonpulsatile or pulsatile cardiopulmonary bypass on endothelial cell permeability was evaluated using the bioassay for endothelial barrier function. Endothelial barrier function is reflected as the overall resistance over the endothelial cell monolayer and the strength of the interaction between endothelial cells (Rb) and between endothelial cells and the matrix (alpha). Figure 6 shows that plasma obtained after cardiopulmonary bypass (post-CPB) induces loss of overall resistance (panel A; RM P<0.001 for nonpulsatile and pulsatile groups) and reduces the strength of the cell-cell interaction (panel B; RM P<0.001 for nonpulsatile and pulsatile groups) and the cell-matrix interaction (panel C; RM P<0.001 for nonpulsatile and pulsatile groups) when compared to baseline values (pre-CPB).

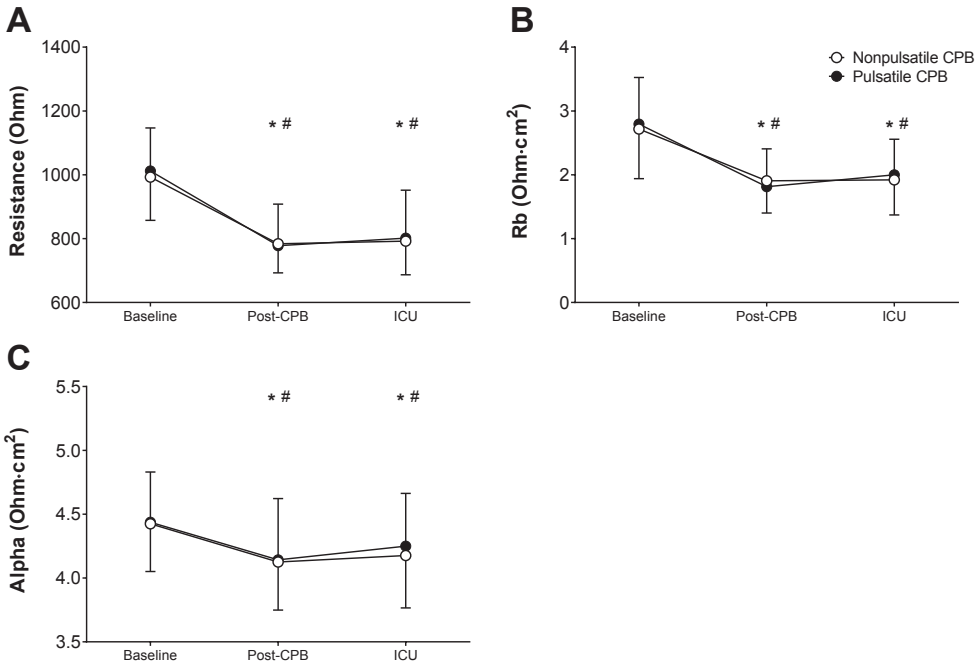

Figure 6 Endothelial barrier function. Endothelial barrier function is represented as overall resistance (panel A), cell-cell interaction (panel B; Rb) and cell-matrix interaction (panel C; alpha) before cardiopulmonary bypass (pre-CPB), after cardiopulmonary bypass (post-CPB) and upon intensive care unit (ICU) admission in nonpulsatile (closed circles) and pulsatile (open circles) flow groups. CPB = cardiopulmonary bypass; ICU = intensive care unit. # P<0.001 vs. pre-CPB values in nonpulsatile flow group; * P<0.001 vs. pre-CPB values in pulsatile flow group.

The negative effect of plasma exposure of endothelial cells on barrier function was still present upon intensive care admission (ICU). The endothelial barrier function parameter mostly affected by exposure to post-CPB plasma was the cell-cell interaction marker, which reduced by 30% and 36% compared to baseline for nonpulsatile and pulsatile flow groups, respectively. The overall resistance and cell-matrix integrity parameter reduced by 21% and 7% in the nonpulsatile flow group, and by 23% and 7% in the pulsatile flow group, respectively. Repeated measures ANOVA revealed no differences in loss of endothelial barrier function between the nonpulsatile and pulsatile CPB groups for overall resistance (RM P=0.839), cell-cell interactions (RM P=0.779) or cell-matrix interactions (RM P=0.639). There were no correlations between clinical parameters or plasma levels of vascular mediators with endothelial barrier function parameters.

Hemodilution

Hemodilution during cardiopulmonary bypass caused a relative decrease in post-CPB hematocrit and hemoglobin levels of approximately 30%, which showed partial restoration upon ICU admission (Table 1). To investigate whether the post-CPB decrease in endothelial barrier function could be explained by CPB-related hemodilution, endothelial cell monolayers were exposed to diluted plasma samples. Figure 7 shows the effects of different concentrations of plasma derived before and after cardiopulmonary bypass on endothelial cellular resistance. The 5x and 10x diluted pre-CPB plasma samples induced the highest increase in overall resistance, followed by 3.3x (30% plasma), 5x (20% plasma) and 10x (10% plasma) diluted post-CPB plasma samples. Dilution of pre-CPB samples did not reduce transendothelial electrical resistance towards post-CPB levels, and a higher fraction of plasma was associated with an improved endothelial barrier function, irrespective of the plasma sampling time point.

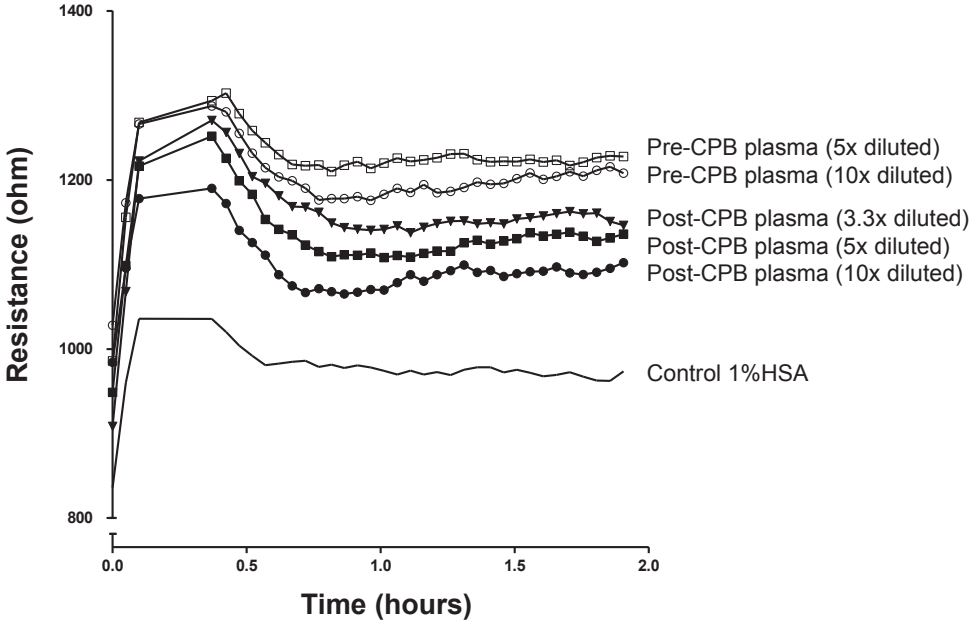

Figure 7 Effect of hemodilution on endothelial barrier function. Changes in resistance following exposure of endothelial cells to different concentrations of plasma, derived before or after cardiopulmonary bypass, and 1% HSA medium. The figure shows five different dilutions of plasma in the medium, the cells were exposed to: two dilutions of the pre-CPB sample (5x and 10x dilution) and three dilutions of the post-CPB sample (3.3x, 5x and 10x dilution). A control sample of 1% HSA medium was used as negative control. Resistances are averaged values of duplicate measurements.

Clinical outcome

Table 2 shows the postoperative outcome parameters. No differences between nonpulsatile and pulsatile groups were observed, nor were there any correlations observed between postoperative outcome and ECIS parameters or plasma markers.

Table 2 Postoperative outcome

Description	Nonpulsatile flow	Pulsatile flow	P-value
Acute kidney injury	2 (10%)	0 (0%)	0.487
Atrial fibrillation	3 (15%)	2 (10%)	1.000
Mortality	1 (5%)	0 (0%)	0.600
Perioperative transfusion (Units; (median (IQR))	1 (0-3)	1 (0-3)	0.522
Extubation within 6 h	16 (80%)	13 (65%)	0.480

Values are displayed as number of events (percentage) or medians (interquartile range). IQR = interquartile range. There were no statistical differences among groups.

Discussion

We show for the first time that onset of cardiopulmonary bypass induced loss of endothelial barrier function in an endothelial monolayer bioassay for trans-endothelial electrical resistance as compared to preoperative levels. The decrease in endothelial barrier function was mainly based on a reduction in endothelial cell-cell interaction. Our study demonstrated that, despite the recognized protective effect of pulsatile blood flow during CPB on microvascular function [8,9,22,23], loss of endothelial barrier function occurred irrespective of the applied flow modality. These findings suggest that CPB-related changes in plasma mediators that affect endothelial barrier function are not prevented by pulsatile flow, and that the protective effects of pulsatile flow should be sought in other mechanisms, such as the local release of endothelial protective mediators by shear stress or direct protection of the endothelial cell layer integrity [21].

Evaluation of the pathophysiology underlying loss of endothelial barrier function in the clinical setting is frequently restricted to the assessment of pulmonary vascular leakage or tissue resistance, which reflects the total of modulating effects of an intervention on vascular permeability [27,28]. Morphological evaluation of endothelial cell cultures exposed to plasma from cardiosurgical patients suggested endothelial hyperpermeability based on increased neutrophil trans-endothelial migration and intracellular gap formation [29], and disruption of endothelial architecture [30], but quantitative analysis of a direct effect of plasma on endothelial barrier integrity was lacking. Others showed that cardiac surgery is associated with increased extravascular lung water, but foremost attributed this association to the effect of allogeneic blood transfusion instead of cardiopulmonary bypass [27,31]. Others showed that an increase in vWF following cardiopulmonary bypass was paralleled by enhanced urine albumin excretion as marker for vascular leakage [32].

The trans-endothelial resistance measurements revealed that the plasma-induced alterations in endothelial barrier function could mainly be attributed to disturbed cell-cell binding, rather than cell-matrix binding. Monolayer hyperpermeability due to alterations in adherens junctions is regulated by, among others, calcium/calmodulin-dependent activation of the myosin light chain kinase and Rho GTPases [33]. Bianchi et al. previously showed that endothelial and cardiomyocyte adherens junctions in atrial and myocardial tissue samples harvested following cardiopulmonary bypass were degraded [17]. Moreover, in a pig model, cardiopulmonary bypass induced a reduction in VE-cadherin, beta-catenin and gamma-catenin levels, suggesting dissociation of the adherens junction complex [18]. Both studies support our findings that cardiopulmonary bypass-induced alterations in endothelial barrier function are mainly explained by disturbed cell-cell interactions.

We assumed that insight in the presence of plasmatic vascular mediators would give clarify the role of these factors in endothelial barrier dysfunction following cardiopulmonary

bypass. Indeed, others showed that cardiopulmonary bypass is associated with an increase in pro-inflammatory mediators, endothelial activation and apoptosis [10-13,34,35], which may contribute to a disturbance in endothelial integrity. We expected that cardiopulmonary bypass would activate the endothelium, leading to an increased availability of the soluble adhesion molecules P-selectin, ICAM-1 and VCAM-1, and an increase in vWF and VEGF as markers for endothelial injury. These molecules additionally exert a direct negative influence on endothelial barrier function [36-38]. In the current study, we indeed found an increase in P-selectin, VCAM-1 and vWF after cardiopulmonary bypass.

Several studies have attributed the Ang/Tie2 system an important role in the development of endothelial hyperpermeability following cardiac surgery [30,39,40]. In line with our findings, Clajus et al. showed that subjecting endothelial monolayers to plasma collected after CPB led to a disturbed endothelial architecture with an increased number of gaps and actin stress fiber [30]. Treatment with recombinant Ang-1 prevented these changes. Moreover, the postoperative increase in plasma Ang-2 levels was associated with respiratory failure in the ICU. Although we were not able to confirm such an association, as we only included low-risk patients, the Ang-2/Ang-1 ratio increased threefold over the study period, demonstrating that the Ang/Tie2 system is disturbed during on-pump cardiac surgery.

Prevention of vascular leakage after cardiac surgery may contribute to preservation of vascular function and prevention of postoperative complications. In addition to experimental substances, like angiopoietin-1 [30,41], there are a few studies that investigated the protective capacity of clinical drugs, including corticosteroids and aprotinin [16,18,42]. Further exploration of these drugs may contribute to the prevention of fluid extravasation following cardiac surgery with cardiopulmonary bypass, and prevent the development of postoperative complications [3].

Although the generalizability of human umbilical vein endothelial cells for central and peripheral vascular networks may be limited, previous studies showed that umbilical endothelial cell cultures are a representable model for human endothelial barrier function [43]. Interestingly, our data show direct activation of the endothelium by circulating factors in the absence of inflammatory cells. The current study provides a first basis whether our bioassay might be used as in vitro read out model for the detection of clinical consequences and complications of increased endothelial permeability related to surgery and extracorporeal circulation.

In conclusion, cardiopulmonary bypass-induced changes in the constitution of human plasma have a profound negative effect on in vitro endothelial barrier function, in particular the cell-cell integrity. The disturbance in endothelial barrier function by human plasma was paralleled by a rise in endothelial activation markers, although it was unrelated to the flow profile used during CPB or the level of hemodilution. The findings in the present study contribute to an extension of our insight in the effects of cardiac surgery with cardiopulmonary bypass on the

permeability of the vasculature. Moreover, the current findings demonstrate that ECIS can be used as a reproducible assay of plasma induced alterations of in vitro endothelial barrier function.

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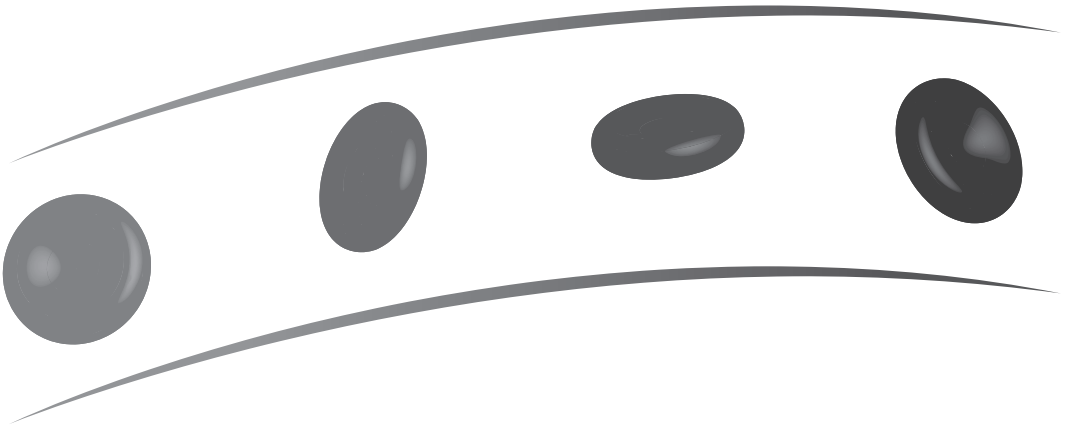

CHAPTER 7

Side-by-side alterations in glycocalyx thickness and perfused microvascular density during acute microcirculatory alterations in cardiac surgery

N.J. Koning, A.B.A. Vonk, H. Vink, C. Boer.

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Abstract

Background: Endothelial glycocalyx injury causes microcirculatory perfusion disturbances in experimental studies, but the relevance in a clinical setting is unknown. We investigated whether glycocalyx dimensions are reduced after onset of cardiopulmonary bypass and whether this is associated with alterations in microvascular perfusion.

Methods: The current observational study included 36 patients undergoing cardiac surgery without or with cardiopulmonary bypass, using either nonpulsatile or pulsatile flow. Sublingual microcirculatory perfusion was assessed perioperatively and analyzed for perfused vessel density (PVD) and perfused boundary region (PBR), an inverse parameter of endothelial glycocalyx dimensions.

Results: Perfused vessel density decreased after onset of cardiopulmonary bypass in parallel with an increase in perfused boundary region in both pulsatile and nonpulsatile groups. In the nonpulsatile CPB group, these alterations were still persistent in the ICU (PVD preoperative: 19.8 ± 2.8 mm/mm^2 vs. postoperative: 15.3 ± 2.6 mm/mm^2; P=0.004. PBR preoperative: 2.40 ± 0.35 µm vs. postoperative: 2.60 ± 0.31 µm; P=0.020). In the off-pump group, perfused vessel density remained unaltered. An inverse correlation between perfused vessel density and perfused boundary region was detected.

Conclusion: The current study shows that endothelial glycocalyx dimensions decrease after onset of cardiopulmonary bypass and are closely related to microvascular perfusion when assessed with a novel, non-invasive technique.

Introduction

Although it is assumed that reduced endothelial glycocalyx integrity is associated with changes in vascular function, including loss of endothelial barrier function [1], inflammation [2] and a prothrombogenic profile [3,4], direct evidence for its role in human pathology and more particularly, microvascular dysfunction, is scarce [5-9]. For a long time it was assumed that systemic blood pressure as driving force was the major determinant of microvascular flow and perfusion. However, microcirculatory flow may be additionally altered by inflammatory activation, temperature, hemodilution and loss of pulsatile flow, irrespective of systemic blood pressure [10-13]. Although there are indications that the integrity of the glycocalyx may also influence vascular perfusion patterns, this has only been shown in experimental animal studies [14-17]. In particular, perturbation of the endothelial glycocalyx causes a reduction in the number of functional capillaries, but it is unknown whether this phenomenon is of importance in a clinical setting [16].

It is commonly recognized that patients undergoing cardiac surgery who are subjected to extracorporeal circulation develop a reduction in sublingual microcirculatory perfusion [12,18]. Moreover, in the absence of use of an extracorporeal circuit, microcirculatory perfusion is largely preserved throughout the surgical procedure [19]. Pulsatile flow during cardiopulmonary bypass has shown to improve postoperative recovery of microvascular density as compared to nonpulsatile flow, which could not be not explained by changes in macrocirculatory parameters as mean arterial blood pressure or cardiac output [12].

We aimed to investigate whether the acute reduction of microcirculatory perfusion during cardiac surgery with cardiopulmonary bypass is associated with alterations in glycocalyx dimensions using a novel in vivo visualization technique of the glycocalyx thickness [8]. We hypothesized that onset of cardiopulmonary bypass reduces endothelial glycocalyx dimensions with a concomitant decrease in perfused microvascular density, and that pulsatile flow improves recovery of both parameters.

Methods

Subjects

The current observational study was approved by the local Human Subjects Committee of the VU University Medical Center (Amsterdam, the Netherlands). Written informed consent was obtained from all subjects. Thirty-six patients undergoing isolated coronary artery bypass surgery with either pulsatile (n=12) or nonpulsatile (n=12) CPB or without cardiopulmonary bypass (off-pump; n=12) between 18 and 85 years of age were included. Exclusion criteria of the current study were previous cardiac surgery, emergency surgery, insulin-dependent diabetes mellitus and a body mass index over 30 kg/m^2. Patients were included after allocation to an on-pump or off-pump technique, for which no selection criteria were applied.

Anesthesia protocol

The anesthesia protocol for all cardiac surgical procedures in our study was similar and earlier described by our group [12]. Briefly, anesthesia was induced using sufentanil, midazolam and pancuronium bromide and a continuous propofol infusion was used for maintenance. After anesthesia induction, all patients received dexamethasone (1 mg/kg) and cefazolin.

In the CPB groups, cardiopulmonary bypass was performed with a S5 heart-lung machine with a heparin coated polyvinyl tubing system (Affinity, Medtronic, Minneapolis, MN, USA). Nonpulsatile or pulsatile CPB (34°C; 2.2-3.0 l/min/m^2) started following heparin administration (300 IU/kg) when the activated clotting time exceeded 480 s. In the pulsatile CPB group, the centrifugal pump used a standard internal algorithm to generate pulsatile flow with a frequency of 60 per minute, as previously described [12].

In off-pump surgery, heparin 300 IU/kg was administered before grafting. Throughout the procedure the activated clotting time was maintained above 380 s. Normothermic temperature management was maintained throughout surgery. At the end of surgery, heparin was reversed with protamine and tranexamic acid was administered in all groups.

Microcirculatory measurements and analysis

Measurements of the sublingual microcirculation using sidestream dark field imaging [20] were performed in all patients after induction of anesthesia (Preoperative), during aortic cross-clamp time on cardiopulmonary bypass or during grafting in off-pump surgery (Intraoperative), and after closure of the sternal incision (Postoperative). At each time point, three recordings of at least 10 seconds were acquired. Care was taken to avoid pressure artifacts during recording. Videos were subsequently analyzed off-line for perfused microvascular density [12] and the perfused boundary region in microvessels ranging from 5 to 25 µm diameters as an inverse measure for glycocalyx thickness.

The perfused boundary region is assessed by an imaging-based digital analysis system (GlycocheckTM glycocalyx measurement software, Glycocheck BV, Maastricht, the Netherlands) that was specifically developed to detect in vivo changes in glycocalyx dimensions in human subjects [8]. The perfused boundary region in microvessels is the red blood cell-poor layer that results from the phase separation between the flowing red blood cells and plasma. The outer edge of the perfused boundary region is defined by the protective part of the glycocalyx that does not allow cell penetration, thereby shielding the endothelial surface and its adhesion molecules from direct contact with circulating cells. Loss of glycocalyx integrity allows for deeper penetration by the outer edge of the red blood cell-perfused lumen and thereby increases the perfused boundary region [3,5,8,9].

Preoperative and intraoperative characteristics

Patient characteristics as age, non-insulin dependent diabetes mellitus and Euroscore (European System for Cardiac Operative Risk Evaluation), a preoperative risk evaluation for patients undergoing cardiac surgery, based on patient characteristics (e.g. age, gender), comorbidities (e.g. renal failure, peripheral vascular disease), cardiac-related factors (e.g. ejection fraction, recent myocardial infarction) and surgery-related factors (e.g. emergency surgery, aortic surgery) [21], were registered. Moreover intraoperative data as temperature, surgical time and for the CPB groups CPB and aortic cross-clamp time was collected.

Statistical analysis

Data were analyzed using SPSS statistical software package (17.0; IBM, New York, USA). All values are expressed as mean ± standard deviation or median with interquartile range (IQR). After testing for normality of distribution, two-way ANOVA's with post-hoc Bonferroni analysis were used to analyze differences between groups. Within-group differences were tested with paired T-tests. Correlations between changes in glycocalyx dimensions and perfused microvascular density were tested with Pearson correlations tests. $P<0.05$ was considered as statistically significant.

Results

Patient and surgical characteristics are shown in Table 1. Although patients included in the pulsatile group were older and tended to have a slightly higher Euroscore, no significant different between groups were detected.

Table 1. Patient characteristics

Description	Off-pump	Nonpulsatile CPB	Pulsatile CPB	P-value
N	12	12	12	NA
Age (years [median (IQR)])	65 (54-65)	65 (61-69)	74 (71-75)	0.518
Diabetes Mellitus II [n] (%)	1 / 12 (8%)	2 / 12 (16%)	2 / 12 (16%)	0.506
Euroscore [median (IQR)]	3 (1-4)	2 (1-3)	4 (3-5)	0.646
Grafts [median (IQR)]	4 (3-5)	3 (3-4)	3 (3-5)	0.144
Surgery time (min)	250 (24)	222 (70)	244 (79)	0.068
CPB time (min)	NA	94 (24)	104 (27)	0.336
Cross-clamp time (min)	NA	60 (18)	73 (18)	0.121
Lowest intraoperative temperature (°C)	36.2 (0.5)	34.2 (0.6)	33.8 (0.4)	0.000

Values are mean (SD) unless otherwise specified. Numeric data were tested with two-way ANOVA for three groups. Nonparametric data were tested with a Kruskal-Wallis test, whereas nominal data were tested with a Chi-square test. CPB = cardiopulmonary bypass, Euroscore = European System for Cardiac Operative Risk Evaluation, IQR = interquartile range, N = number, NA = Not applicable.

Patients undergoing off-pump surgery show a stable perfusion of the microcirculation, as represented by a perfused vascular density (PVD) of 19.8 ± 2.8 mm/mm^2, 20.3 ± 2.4 mm/mm^2 and 19.4 ± 2.3 mm/mm^2 at the preoperative, intraoperative and postoperative time points, respectively (Figure 1A; NS between time points). Both groups undergoing CPB demonstrated a decrease in perfused vessel density intraoperatively as compared to the off-pump group (nonpulsatile CPB 15.1 ± 2.7 mm/mm^2, P< 0.001 vs. off-pump; pulsatile CPB 16.7 ± 2.6 mm/mm^2, P=0.002 vs. off-pump). Whereas nonpulsatile cardiopulmonary bypass is associated with prolonged impairment of microcirculatory perfusion until intensive care unit admission, administration of pulsatile flow during extracorporeal circulation with centrifugal pumps restored microcirculatory perfusion in the postoperative period (Figure 1A; repeated measures ANOVA for between-group differences P=0.02).

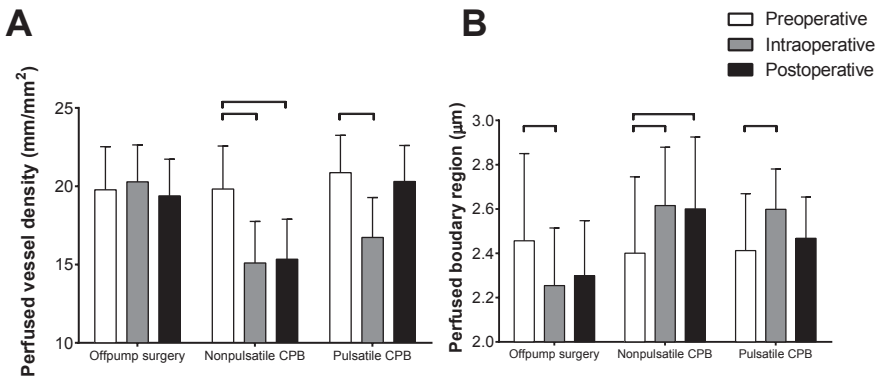

Figure 1. Perioperative microvascular perfusion and endothelial glycocalyx dimensions. Perfused vessel density (Panel A) decreases from the preoperative to the intraoperative time point in both CPB groups, along with increases in perfused boundary region (Panel B). Postoperatively, only the nonpulsatile CPB group demonstrates decreased perfused vessel density and increased perfused boundary region. Both parameters were tested with two-way ANOVA's with post-hoc Bonferroni analysis. Parentheses indicate P<0.05 as tested with post-hoc Bonferroni tests.

The microvascular perfused boundary region in patients undergoing off-pump surgery decreased throughout the intraoperative period (Preoperative: 2.46 ± 0.40 µm, Intraoperative: 2.25 ± 0.26 µm; P=0.030 vs. Preoperative, Postoperative: 2.30 ± 0.25 µm; NS vs. Preoperative), suggesting that the glycocalyx dimensions increased (Figure 1B). In the on-pump groups, the perfused boundary region primarily increases after initiation of cardiopulmonary bypass (nonpulsatile CPB 2.60 ± 0.26 µm, P=0.003 vs. off-pump; pulsatile CPB 2.60 ± 0.18 µm, P=0.001 vs. off-pump), and remained increased postoperatively only in the nonpulsatile flow group (2.59 ± 0.31µm, P<0.001 vs. both off-pump and pulsatile CPB).

Figure 2 shows a moderate to good association between the perfused boundary region with microcirculatory perfusion (perfused vessel density; r=-0.65; P=0.002).

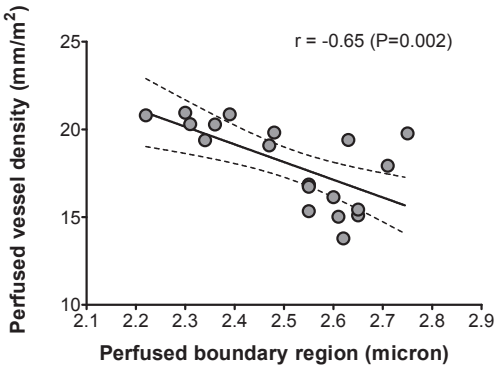

Figure 2. Scatterplot between perfused boundary region and perfused vessel density. Decreasing endothelial glycocalyx dimensions are associated with reduced perfused microvascular density. Data was tested with a Pearson correlation test.

Discussion

In the current article, we show that glycocalyx dimensions are reduced after onset of cardiopulmonary bypass in patients undergoing cardiac surgery, in contrast to off-pump surgery. Moreover, we demonstrate for the first time the close relation between acute glycocalyx perturbation and microcirculatory perfusion disturbances in human subjects. In line with our hypothesis, pulsatile flow during CPB was associated with recovery of glycocalyx dimensions and microvascular perfusion postoperatively, where this recovery was absent after nonpulsatile CPB.

Previously, Brueger et al. have demonstrated that plasma levels of endothelial glycocalyx components augment to a higher extent during on-pump as compared to off-pump cardiac surgery [22]. Our current data support the observations that onset of cardiopulmonary bypass is associated with acute glycocalyx injury, whereas off-pump cardiac surgery was not associated with reduced glycocalyx dimensions or with a reduction in perfused vessel density. Additionally, we demonstrate the dynamic nature of the endothelial glycocalyx. In patients undergoing pulsatile cardiopulmonary bypass, glycocalyx dimensions restored within 4 hours after primary injury by onset of extracorporeal circulation. It appears that, in the presence of sufficient shear stress [23], potential glycocalyx substrates as fresh plasma, albumin or heparin derivates [24-27], the endothelium is capable to restore its glycocalyx when exposed to physiological circumstances. In contrast, following nonpulsatile flow, we did not observe glycocalyx dimensions that restored as compared to baseline in the current study window. It is hypothesized that disturbed endothelial function by nonpulsatile flow and concomitantly reduced peak shear stress [28] prevents recovery of microvascular perfusion after extracorporeal circulation. This implies that the conditions to which the vascular endothelium is exposed, are not only relevant for the initial disturbance of glycocalyx, but also for endothelial glycocalyx restoration after primary injury.

The restoration capacity of the endothelial glycocalyx is additionally demonstrated by an increase in glycocalyx dimensions from the preoperative to the intraoperative period in the off-pump group. Although speculative, a possible explanation may be sought in the effect of heparin, which is administered after the preoperative measurement. Unfractionated heparin may inhibit heparanase activity and therefore protect the endothelial glycocalyx [26-27]. Although the CPB groups receive identical initial heparin doses, the onset of CPB with concomitant effects may have masked the beneficial effect of heparin on the endothelial glycocalyx.

Perturbation of endothelial glycocalyx in experimental models leads to microcirculatory perfusion disturbances [16]. This is most likely to be attributed to a combination of increased exposure of endothelial adhesion molecules and increased vascular leakage [29,30]. Consequently, leukocytes, thrombocytes and erythrocytes may adhere to the microvascular

endothelium and perivascular pressure will rise, respectively, both of which might compromise patency of microvessels [16]. Additionally, of the arteriolar side of the microvascular bed, the endothelial glycocalyx contributes to shear stress transduction to the endothelial cells and subsequent eNOS activation [31]. Disturbances of this function might further compromise microvascular perfusion, particularly in the face of reduced peak shear stress levels during nonpulsatile cardiopulmonary bypass [28]. The causal relationship between changes in glycocalyx integrity and intravascular flow profiles is currently unclear. Loss of endothelial glycocalyx volume may lead to reduced intravascular resistance and increased capillary blood flow, albeit that this increase in blood flow is not necessarily beneficial. We have previously demonstrated that in patients undergoing nonpulsatile cardiopulmonary bypass, a fraction of capillaries exerts very high blood velocities during and after CPB, which was associated with functional shunting of oxygen [32]. Simultaneously, increased heterogeneity of microvascular perfusion was observed, whereas this was not detected during off-pump surgery [32]. Inversely, it might be conceivable that the more fragile part of the microcirculation becomes unprotected due to loss of vascular autoregulation during CPB, which may consequently lead to endothelial glycocalyx damage due to high RBC flow rates [33].

The off-pump group remained normothermic, while the CPB groups became mildly hypothermic due to temperature drifting. The direct effects of mild hypothermia during CPB on microcirculatory perfusion and the endothelial glycocalyx are currently unknown [34]. Although a reduction in oxygen consumption and possibly microcirculatory perfusion might be expected with hypothermia, rewarming after CPB should lead to a recovery of the microcirculation. Unfortunately, no correlations between temperature and either microvascular or glycocalyx parameters could be detected in the current study, and the effects of mild hypothermia on the microcirculation remain therefore undetermined.

A limitation of the current study is the indirect measurement of endothelial glycocalyx dimensions. In particular, the applied microvascular imaging technique measures changes in the intravascular perfused boundary region, which is inversely related to glycocalyx thickness. Since the endothelial glycocalyx is very fragile and its clinical investigation is often invasive, the current literature is largely confined to experimental studies. The technique as applied in this study has been developed in order to enable clinical endothelial glycocalyx research, and the technique proved valuable in multiple patient populations [5-9].

Although the current investigation cannot prove a causal effect between glycocalyx injury and consequent microvascular perfusion disorders, the current technique used to investigate glycocalyx dimensions makes an inverse relation unlikely since glycocalyx dimensions are only determined in the remaining capillaries supporting red blood cells. The current results provide a strong argument for a mediating role of glycocalyx damage in the loss of perfused capillaries in the setting of acute inflammatory activation in humans. Moreover, these observations fortify experimental evidence that glycocalyx disruption is followed by

reduced microvascular perfusion in the absence of macrovascular alterations [16] and that reduced endothelial glycocalyx dimensions in septic patients were associated with increased leukocyte-endothelial interactions [7]. Additionally, in vivo observations in humans suggest that chronic glycocalyx disturbances are associated with reduced renal function [8] and with reduced microvascular perfusion in a population study with middle-aged volunteers [9].

In conclusion, the current study demonstrates that glycocalyx degradation is closely related to acute impairment of microvascular perfusion in a clinical setting. In human patients undergoing cardiac surgery, onset of cardiopulmonary bypass is an important stimulus of acute glycocalyx degradation. Interestingly, in the presence of pulsatile but not nonpulsatile flow during cardiopulmonary bypass, the vascular endothelium was capable to achieve an early glycocalyx restoration. The role of the endothelial glycocalyx in human microvascular disorders has often been overlooked, probably due to its sensitive nature and the complexity of its investigation, both in vivo and ex vivo. The technique currently used may prove to be an important step towards better understanding of the relevance of the endothelial glycocalyx and its injurious stimuli due to a direct and non-invasive investigation of glycocalyx dimensions.

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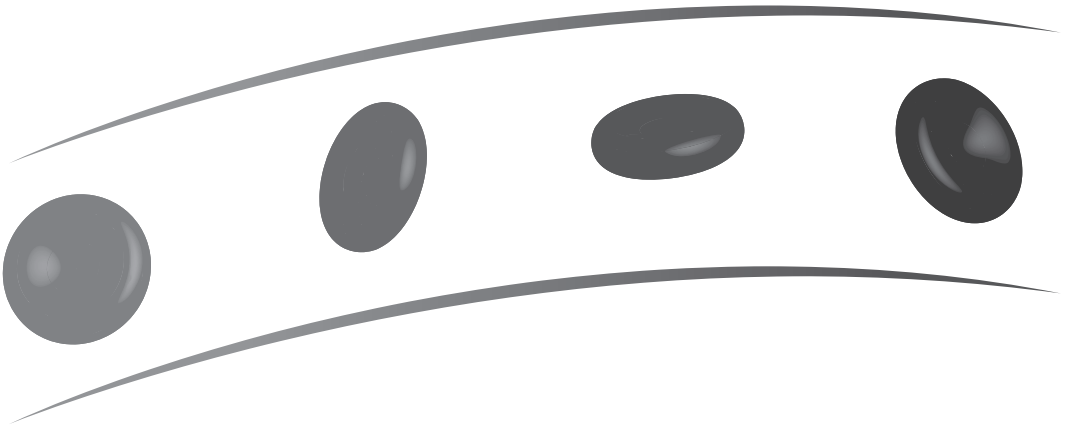

CHAPTER 8

Impaired microcirculatory perfusion in a rat model of cardiopulmonary bypass: the role of hemodilution

N.J. Koning, F. de Lange, A.B.A. Vonk, Y. Ahmed, C.E. van den Brom, S. Bogaards, M. van Meurs, R.M. Jongman, C.G. Schalkwijk, M.P.V Begieneman, H.W.M. Niessen, C. Baufreton, C. Boer.

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Abstract

Background: Although hemodilution is attributed as the main cause of microcirculatory impairment during cardiopulmonary bypass (CPB), this relationship has never been investigated. We investigated the distinct effects of hemodilution with or without CPB on microvascular perfusion and subsequent renal tissue injury in a rat model.

Methods: Male Wistar rats (375-425 g) were anesthetized, prepared for cremaster muscle intravital microscopy and subjected to CPB (n=9), hemodilution alone (n=9) or a sham procedure (n=6). Microcirculatory recordings were performed at multiple time points and analyzed for perfusion characteristics. Kidney and lung tissue were investigated for mRNA expression for genes regulating inflammation and endothelial adhesion molecule expression. Renal injury was assessed with immunohistochemistry.

Results: Hematocrit levels dropped to 0.24 ± 0.03 l/l and 0.22 ± 0.02 l/l after onset of hemodilution with or without CPB. Microcirculatory perfusion remained unaltered in sham rats. Hemodilution alone induced a 13% decrease in perfused capillaries, after which recovery was observed. Onset of CPB reduced the perfused capillaries by 40% (9.2 ± 0.9 to 5.5 ± 1.5 perfused capillaries per microscope field; $P<0.001$), and this reduction persisted throughout the experiment. Endothelial and inflammatory activation and renal histological injury were increased after CPB compared with hemodilution or sham procedure.

Conclusion: Hemodilution leads to minor and transient disturbances in microcirculatory perfusion, which cannot fully explain impaired microcirculation following cardiopulmonary bypass. CPB led to increased renal injury and endothelial adhesion molecule expression in the kidney and lung compared with hemodilution. Our findings suggest that microcirculatory impairment during CPB may play a role in the development of kidney injury.

Introduction

Microcirculatory perfusion is impaired during and after cardiac surgery with cardiopulmonary bypass (CPB). We have previously shown that onset of CPB results in reduction of microcirculatory perfusion [1], and increased heterogeneity in the distribution of microcirculatory perfusion [2], which may lead to disturbances in tissue oxygenation and subsequent tissue injury [3-5].

Since impairment of microcirculatory perfusion is a negative predictor of outcome in critically ill patients [6] and is thought to play an important role in the development of organ dysfunction [7-9], insight in the mechanisms underlying perioperative microvascular dysfunction is warranted. Many factors related to cardiopulmonary bypass play a role in microcirculatory disturbances at the initiation of CPB, but hemodilution is attributed a central role [10-12]. We previously showed preserved microvascular perfusion throughout the perioperative period in patients undergoing off-pump cardiac surgery without hemodilution [12]. Findings from experimental studies showed that acute normovolemic hemodilution resulted in a reduction of perfused vessel density [13], increased endothelial activation [14], and impaired renal oxygenation [15]. In addition, an increase in hematocrit by red blood cell transfusions during CPB improved microcirculatory perfusion in cardiosurgical patients [16,17]. These findings suggest that hemodilution itself is associated with disturbances in microcirculatory perfusion. It is however unknown whether hemodilution is explanatory for all of the detrimental effects of CPB on the microcirculation, as CPB induces an additional inflammatory and endothelial activation that may contribute to microcirculatory impairment [18].

This study therefore investigated the role of hemodilution in the impairment of microcirculatory perfusion observed during cardiopulmonary bypass in a rat model using intravital microscopy. We hypothesized that the deteriorating effects of CPB on microcirculatory perfusion are not completely explained by hemodilution, but additionally involve systemic inflammation and endothelial activation.

Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the VU University, and were conducted following the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the Dutch Animal Experimentation Act. Male Wistar rats of 375–425 g (Charles River Laboratories, Brussels, Belgium) were housed four per cage with unrestricted access to food and water in a temperature-controlled room (20–23°C; 40–60% humidity) with a 12/12 h light dark cycle. Animal care was performed according to the national guidelines for care of laboratory animals. Twenty-four rats were randomly allocated to undergo a cardiopulmonary bypass (n=9), hemodilution (n=9) or sham protocol (n=6). Additionally, six rats were sacrificed immediately after induction of anesthesia for baseline assessment of RT-PCR analyses.

Anesthesia and surgery

Anesthesia was induced with 5% isoflurane in 100% oxygen followed by endotracheal intubation with a 16G catheter (Venflon Pro, Becton Dickinson, Helsingborg, Sweden). Volume controlled mechanical ventilation (UMV-03, UNO Roestvaststaal BV, Zevenaar, The Netherlands; tidal volume 10ml/kg, ventilator frequency 60/min, PEEP 2-4 cmH2O) was initiated with 2-3% isoflurane in an oxygen/air mixture with an FiO_2 of 0.35. Rats were placed on a heating pad, the rectal temperature probe was inserted to maintain body temperature at 36.5 °C and ECG electrodes were connected.

The tail artery was cannulated with a 22G catheter (Venflon Pro, Becton Dickinson, Helsingborg, Sweden) for continuous arterial blood pressure monitoring. Fentanyl boluses (12 µg/kg, Janssen-Cilag, Tilburg, the Netherlands) were then administered intermittently at set time points in all groups, followed by a reduction of inhaled isoflurane concentration (1.5-2.0%). The left cremaster muscle was isolated under warm saline superfusion according to the technique previously described by Baez [19], and with covered gas impermeable plastic film (Saran Wrap, presoaked for 24 hours in distilled water). Heparin (500 IU/kg, LEO Pharma, Amsterdam, The Netherlands) was administered. Subsequently, the right femoral artery was cannulated with a 20G catheter (Arterial Cannula, Becton Dickinson, Helsingborg, Sweden) for arterial inflow of the CPB circuit. Arterial blood gas analysis (Radiometer ABL-50, Radiometer, Brønshøj, Denmark) and hematocrit measurement were performed at baseline and repeated at 10, 30 and 60 minutes of extracorporeal circulation and 10 and 60 minutes after discontinuation of CPB, or at corresponding time points for hemodilution or sham experiments. Before initiation of a study protocol, a repeat dose heparin (500 IU/kg) was given, in combination with pancuronium bromide (0.5 mg/kg, Organon, Oss, the Netherlands). Rats were sacrificed and renal and pulmonary tissue was harvested directly after the last microcirculatory recordings.

Hemodilution protocol

The right jugular vein was cannulated with a 18G catheter (Venflon Pro, Becton Dickinson, Helsingborg, Sweden). Six ml venous blood was withdrawn and exchanged simultaneously with 6 ml 6% hydroxyethyl starch (HES 130/0.4; Voluven, Fresenius Kabi, Halden, Norway). Hematocrit was controlled 10 minutes after hemodilution, additional blood was exchanged if hematocrit levels were higher than 0.25 l/l, to aim for a hematocrit of 0.22 to 0.24 l/l. Ventilation and temperature settings remained unaltered as compared to baseline. In concordance with the CPB-group, protamine hydrochloride (2 mg/kg, Meda Pharma BV, Amstelveen, The Netherlands) was infused 90 minutes after initial hemodilution.

Cardiopulmonary bypass protocol

The protocol for cardiopulmonary bypass was based on a previous report [20]. The CPB circuit, as previously described, consisted of a Plexiglas open venous reservoir, a roller pump (Pericor SF70, Verder, Haan, Germany), and a 4 ml Plexiglas oxygenator-heat exchanger (Ing. M. Humbs, Valley, Germany) with a three layer hollow fiber membrane (Oxyphan, Membrana, Wuppertal, Germany) for gas exchange [21]. A 1.0 mm diameter arterial line (LectroCath, Vygon, Ecouen, France) was connected to the femoral inflow catheter. The circuit was primed with 10 ml of 6% HES.

After cannulation of the right jugular vein with a modified multi-orifice 4.5 French catheter (Desilets-Hoffman, Cook, Bloomington, IN, USA) that was advanced into the right atrium, CPB was initiated. Flow rates of 150-200 ml/kg/min were maintained during extracorporeal circulation, corresponding with 100% of the normal rat cardiac output [22]. The venous cannula was positioned to minimize residual blood flow through the heart, which was associated with minimal residual arterial pulsations. Ventilation was discontinued, a mixture of oxygen and carbon dioxide was led through the oxygenator membrane to maintain pO_2 values between 150 and 250 mmHg and pCO_2 levels between 32 and 45 mmHg. Isoflurane (1.0-1.5%) was added to the gas mixture. Temperature was maintained between 35.0 and 35.5°C. At 65 minutes of CPB, ventilation was restarted at a frequency of 30/min and rats were rewarmed to 36.5°C. Weaning from CPB occurred after 75 minutes of extracorporeal circulation, ventilation frequency was increased to 60/min. The venous cannula was removed and the jugular vein was clamped. Protamine hydrochloride (2 mg/kg) was administered to neutralize heparin 15 minutes after weaning from CPB.

Sham protocol

Instrumentation of rats undergoing a sham procedure was identical to the hemodilution group, including heparin and protamine administration. No other interventions were made throughout the procedure, and all analyses occurred identical as in the other protocols.

Intravital microscopy

The microvasculature of the cremaster muscle was observed with a 10x objective (WN-Achroplan, Zeiss, Oberkochen, Germany; numerical aperture 0.30) on an intravital microscope (AxiotechVario 100HD, Zeiss) connected to digital camera (scA640, Basler, Ahrensburg, Germany). Final magnification was 640x. Three regions of adequate tissue quality and perfusion were selected for baseline and all of the subsequent measurements. In each region, four videos of 10 to 15 seconds were recorded, so that twelve videos per time point were obtained. The camera was aligned to observe capillaries in a horizontal fashion on the screen. Baseline measurements were performed after a 30-minute stabilization period of the cremaster muscle. Subsequent measurements were made at 10, 30 and 60 minutes of CPB and 10 and 60 minutes after discontinuation of CPB, or corresponding time points in other groups.

Microcirculation measurements

Microcirculatory analyses were performed off-line and the investigator was blinded for the allocated treatment protocol. Similar to previously described methods, two vertical test lines were drawn on the screen, on which the capillary crossings were counted and averaged to obtain number of vessels per video screen [23]. Vessels were subdivided in continuously perfused, intermittently perfused and nonperfused capillaries [24]. Spatial heterogeneity of microcirculatory perfusion was assessed by calculation of the coefficient of variation (SD/mean) of the number of perfused vessels over the twelve recording per time point.

Gene expression analysis by real time RT-PCR

Total RNA was isolated from cryosections from kidney and lung and isolated using the RNeasy Mini Plus Kit (Qiagen; Westburg, Leusden, The Netherlands) according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoresis. RNA yield and purity were measured with NanoDrop®ND-1000 UVvis spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA was reverse-transcribed in cDNA using SuperScript®III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). The following Assays-on-Demand primers (Applied Biosystems Systems, Foster City, CA) were used for quantitative PCR: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; assay ID Rn01775763_g1), E-selectin (assay ID Rn00594072_m1), P-selectin (assay ID Rn00565416_m1), intercellular adhesion molecule-1 (ICAM-1; assay

ID Rn00564227_m1), interleukin-6 (IL-6; assay ID Rn00561420_m1), interleukin-10 (IL-10; assay ID Rn00563409_m1), tumor necrosis factor-α (TNF-α; assay ID Rn00562055_m1) and vascular cell adhesion molecule-1 (VCAM-1; assay ID Rn00563627_m1). Samples were run in duplicate and the obtained threshold cycle values (CT) were averaged. Gene expression was normalized to the expression of the housekeeping gene GAPDH (ΔCT). mRNA levels relative to GAPDH were calculated by 2-ΔCT values and averaged per group.

Immunohistochemical analysis

Renal immunohistochemical analysis was performed in order to assess glomerular neutrophil infiltration with myeloperoxidase (MPO) staining and for detection of an inflammatory status of the glomerular endothelium with N(epsilon)-(carboxymethyl)lysine (CML) staining [25,26]. Following termination of the experiments, the left kidney was harvested and preserved in 4% formalin, embedded in paraffin and cut into 4 µm thick sections. The sections were dewaxed, rehydrated and incubated in methanol/H_2O_2 (0.3%) for 30 minutes to block endogenous peroxidases. Next, antigen retrieval was performed either by heat inactivation in citrate buffer (pH 6.0; for MPO staining), or enzymatic (for CML staining) at 37°C for 30 minutes using pepsine-HCl 0.1% solution. Slides to be stained with CML were incubated with normal rabbit serum (1:50, Dako) for 10 minutes at room temperature (RT), as previously described [27]. Next, slides were incubated with either mouse-anti-rat MPO (1:50, Abcam, Cambridge, UK) or CML (1:2000) for 1 hour at RT. Followed by incubation with Envision (MPO slides, undiluted, anti-mouse/rabbit, Dako) or rabbit-anti-mouse-biotine (CML slides, 1:500, Dako) for 30 minutes at RT. CML slides were then incubated with streptavidine-horseradish peroxidase (1:100, Dako) for 1 hour at RT. Finally, all sections were visualized using 3,3'-diaminobenzidine (0.1 mg/ml, 0.02% H_2O_2) and counterstained with hematoxylin, dehydrated and covered. With each staining a PBS control was included. All these controls yielded negative results (data not shown).

In anti-MPO stained sections, the number of deposed neutrophils per glomerulus were counted for 100 glomeruli per section. Analysis of CML-stained sections occurred as previously described [25]. In brief, endothelial cell positivity was assessed semi-quantitatively using scores ranging from 0 (no positivity) to 3 (strong positivity). The average of 100 glomeruli was used as intensity score for the section. The investigator performing the analysis was blinded for group allocation.

Statistical analysis

Data were analyzed using a SPSS statistical software package (IBM, version 17.0). All values are expressed as mean ± standard deviation or median with interquartile range (IQR). Normality of distribution was tested with the Shapiro-Wilk test. Repeated-measures (RM) ANOVA was performed to analyse time-dependent differences between groups. For parameters that

were normally distributed, two-way ANOVA tests with post-hoc Bonferroni comparisons were used to compare between groups at individual time-points, whereas group-dependent changes from baseline values were analyzed by a paired T-test. Kruskal-Wallis tests with post-hoc Bonferroni comparisons and Wilcoxon tests were used to evaluate differences in nonparametric data between groups and within groups, respectively. $P < 0.05$ was considered as statistically significant.

Results

The rats weighed 393 g [372-412], 415 g [386-419] and 410 g [392-424] for sham, hemodilution and CPB groups, respectively (P=0.286). All animals completed the experimental protocol and were analyzed on an intention to treat basis.

Systemic variables

In the hemodilution and CPB group, we aimed for a similar drop in hematocrit levels. Baseline hematocrit (0.43 ± 0.04 l/l vs. 0.43 ± 0.02 l/l; P=0.870) and hematocrit values at 10 minutes following initiation of CPB (0.24 ± 0.03 l/l vs. 0.22 ± 0.02 l/l; P<0.001 vs. baseline for both groups; P=0.122 between groups) were comparable between the hemodilution and CPB groups, respectively (Figure 1A). In the sham group, hematocrit levels remained unaltered throughout the experiment (ANOVA RM within group effect: P=0.335). There was a transient decrease in mean arterial pressure during hemodilution and CPB, but this returned back to baseline values towards the end of the experiment (Figure 1B).

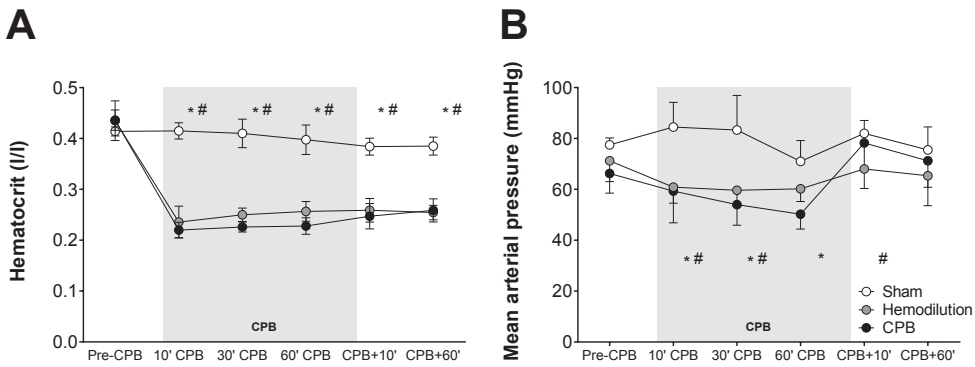

Figure 1 Systemic variables. Course of hematocrit (Panel A) and mean arterial blood pressure (Panel B) during the experiments for the sham group (white dots), hemodilution group (grey dots) and CPB group (black dots). The x-axes were labelled according to events in the CPB group, the grey rectangles in the background depict the period of extracorporeal circulation in the CPB group. Similar levels of hemodilution were obtained in both study groups. *P<0.05 CPB vs Sham, #P<0.05 Hemodilution vs Sham.

Microcirculatory perfusion during and after CPB

In the sham group, microcirculatory perfusion did not change during the experimental protocol (Figure 2A). Hemodilution per se led to an initial reduction in perfused capillaries of 13% (from 8.8 ± 0.6 to 7.7 ± 1.1 perfused capillaries per microscope field; P=0.028). This transient reduction in microcirculatory perfusion was only observed in the first two measurements after hemodilution, followed by restoration of the microcirculation to baseline values. Onset of cardiopulmonary bypass induced a 40% decrease in the number of perfused microvessels (from 9.2 ± 0.9 to 5.5 ± 1.5 perfused capillaries per microscope field; P<0.001).

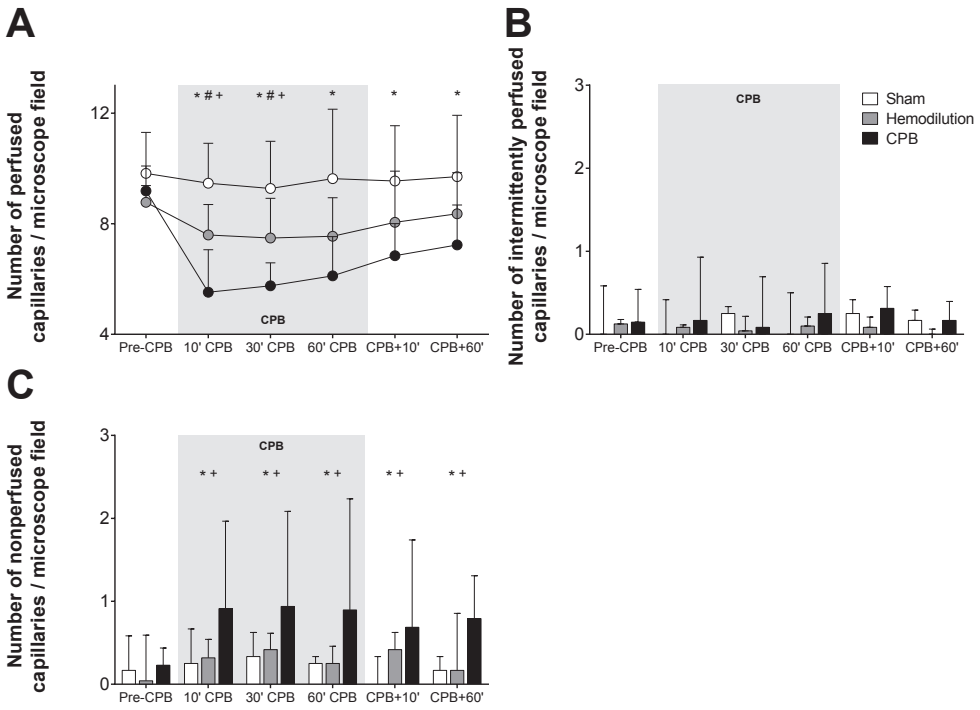

Figure 2 In vivo cremaster muscle microvascular perfusion. Number of perfused (Panel A), intermittently perfused (Panel B) and nonperfused (Panel C) vessels in the sham group (white), hemodilution group (grey) and CPB group (black). The x-axes were labelled according to events in the CPB group, the grey rectangles in the background depict the period of extracorporeal circulation in the CPB group. Onset of CPB leads to a major decrease in perfused vessels and an increase in nonperfused vessels, whereas acute hemodilution yields a minor and transient decrease in perfused vessels. Microcirculatory perfusion remained unaltered in the sham group. Data is presented as mean values with standard deviation in panel A and as median values with 75th percentile in the bar graphs of panel B and C. *P<0.05 CPB vs Sham, #P<0.05 Hemodilution vs Sham, +P<0.05 Hemodilution vs CPB.

The impairment of microcirculatory perfusion persisted at all measurements during and after extracorporeal circulation. At one hour after weaning from CPB, a 22% decrease in perfused capillaries as compared to baseline was still observed (7.2 ± 1.4 perfused capillaries per microscope field; P=0.01 vs baseline).

No differences between groups in intermittently perfused vessels (Figure 2B) could be detected. Hemodilution alone or the sham procedure did not increase the number of nonperfused microvessels at any time point (Figure 2C). However, immediately after initiation of cardiopulmonary bypass a fourfold increase in nonperfused microvessels was observed (from 0.2 [0.1-0.4] to 0.9 [0.5-2.0] nonperfused capillaries per microscope field; P=0.014). The number of stagnant capillaries remained increased, even after disconnection from cardiopulmonary bypass.

Spatial heterogeneity of microcirculatory perfusion did not alter as compared to baseline in the hemodilution group or in the sham group. In the CPB group, spatial heterogeneity increased after onset of extracorporeal circulation (Coefficient of variation: 0.23 ± 0.05 to 0.44 ± 0.18; P=0.011) and remained increased throughout the study period.

Renal and pulmonary inflammatory cytokine and endothelial adhesion molecule expression during and after CPB

Levels of renal and pulmonary pro- and anti-inflammatory cytokines were assessed using real time RT-PCR are presented in Figure 3. CPB increased TNF-α (Figure 3A and B) and IL-6 (Figure 3C and D) mRNA expression in both kidney and lung tissue as compared to the other groups (ANOVA P<0.001 for both renal and pulmonary TNFα, IL-6 and IL-10-mRNA expression). Hemodilution alone was associated with increased pulmonary TNF-α mRNA expression as compared to baseline only. Additionally, the anti-inflammatory cytokine IL-10 mRNA expression (Figure 3E and F) was increased in the kidneys and lungs in the CPB group only.

The early endothelial adhesion molecules P-selectin mRNA (Figure 4A) and E-selectin mRNA (Figure 4C) showed increased expression after CPB in the kidney as compared to all other groups (ANOVA P<0.001 for renal P-selectin and E-selectin mRNA expression). In lung tissue, E-selectin mRNA was increased after CPB as compared to baseline and sham group (Figure 4D) whereas P-selectin mRNA expression was higher in both CPB and hemodilution groups than in sham and baseline groups (Figure 4B; ANOVA P<0.001 for pulmonary P-selectin and E-selectin mRNA expression). ICAM-1 mRNA (Figure 4E) and VCAM-1 mRNA (Figure 4G) were increased in the CPB group as compared to all other groups in renal tissue (ANOVA P<0.001 for renal ICAM-1 and VCAM-1 mRNA expression). In pulmonary tissue, ICAM-1 demonstrated increase in only the CPB group (Figure 4F), whereas VCAM-1 was at similarly high levels in both the hemodilution and CPB groups (Figure 4H; ANOVA P<0.001 for pulmonary ICAM-1 and VCAM-1 mRNA expression).

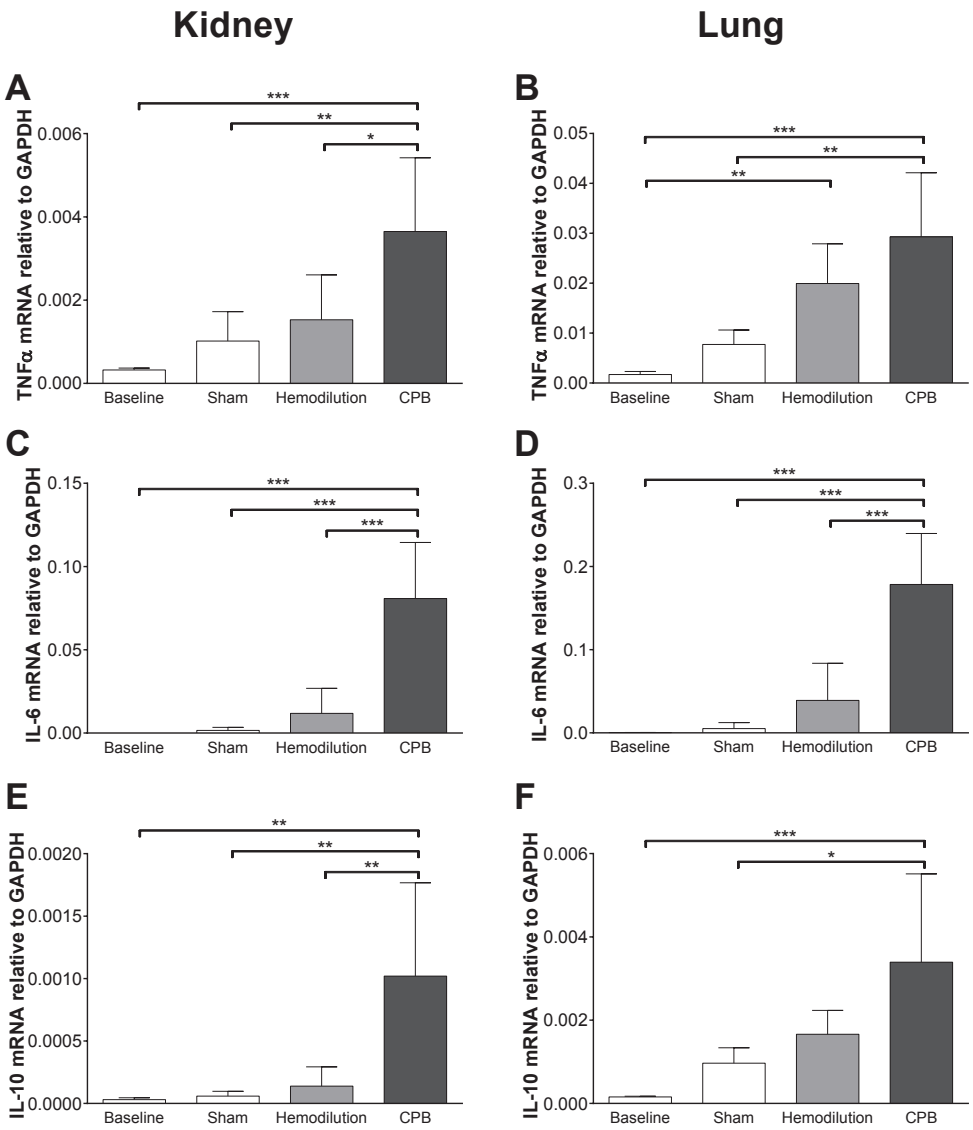

Figure 3 Renal and pulmonary inflammatory cytokine expression mRNA expression of the pro-inflammatory markers TNF-α (Panel A and B), IL-6 (Panel C and D) and the anti-inflammatory IL-10 (Panel E and F) assessed in kidney and lung tissue. CPB was associated with increased pro- and anti-inflammatory activation as compared to the other groups. *P<0.05, **P<0.01 and ***P<0.001 between groups, as tested with Bonferroni post-hoc tests.

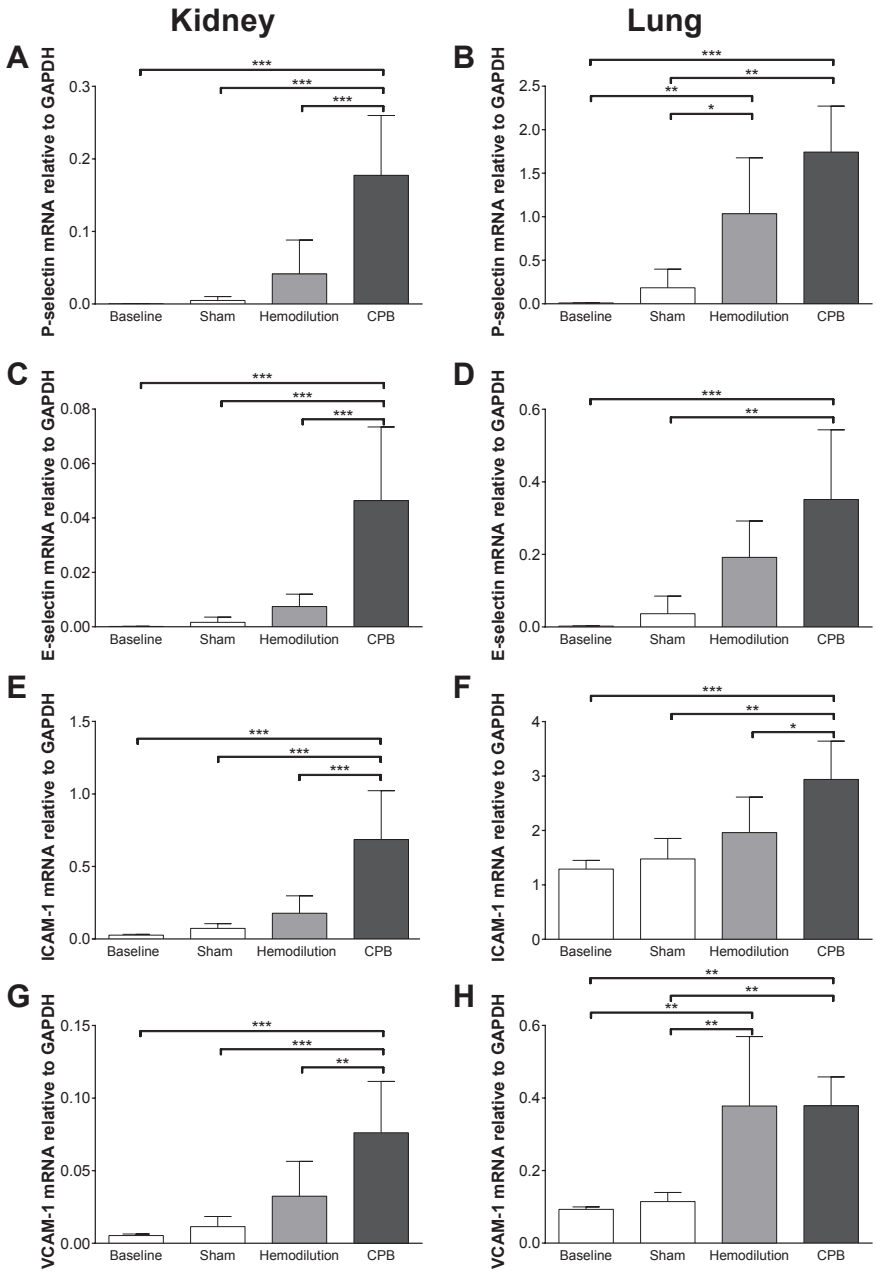

Figure 4 Renal and pulmonary endothelial adhesion molecule expression mRNA expression of the endothelial activation markers P-selectin (Panel A and B), E-selectin (Panel C and D) ICAM-1 (Panel E and F) and VCAM-1 (Panel G and H) assessed in kidney and lung tissue. CPB was associated with increased endothelial activation as compared to the other groups, whereas the hemodilution group showed increased activation of P-selectin and VCAM-1 relative to the sham and baseline groups. *P<0.05, **P<0.01 and ***P<0.001 between groups, as tested with Bonferroni post-hoc tests.

Renal damage during and after CPB

In glomeruli subjected to CPB, a higher neutrophil influx was detected in renal sections stained with anti-MPO as compared to following hemodilution or sham procedures (Figure 5G; Kruskal-Wallis test P=0.001). CPB led to increased renal glomerular endothelial inflammation as compared to hemodilution and sham groups (Figure 5H; Kruskal-Wallis test P=0.001).

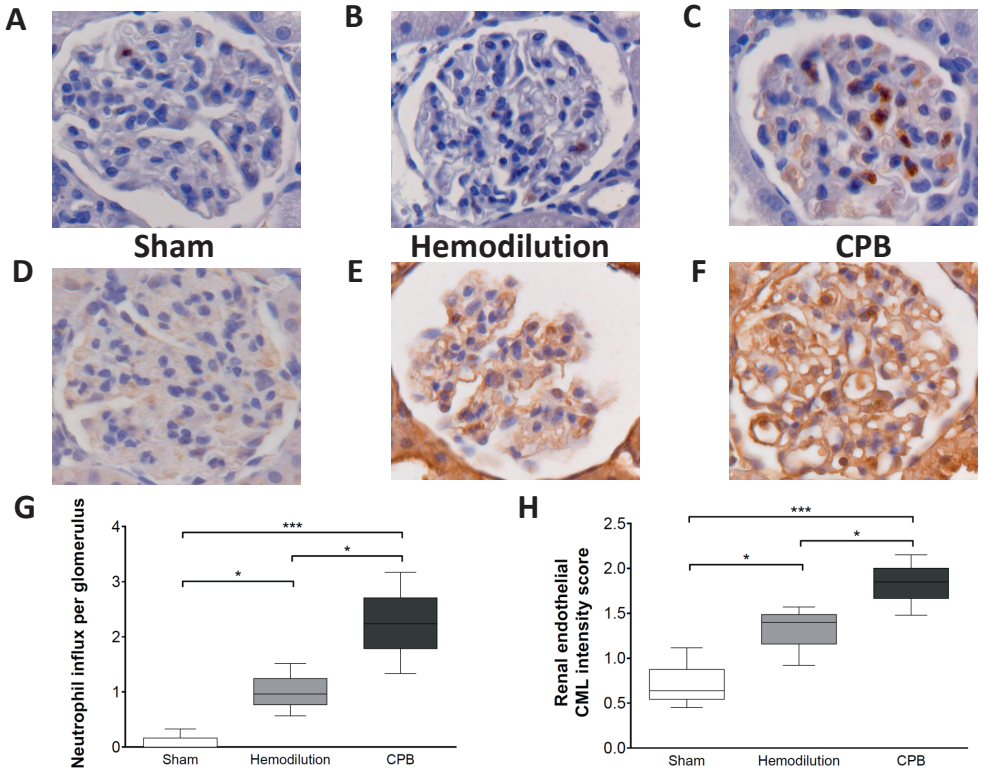

Figure 5 Renal immunohistological analyses Renal sections were stained with antibodies against myeloperoxidase (MPO) and N(epsilon)-(carboxymethyl)lysine (CML) and analyzed for glomerular neutrophilic deposition and CML depositions of the glomerular endothelium, respectively. Typical examples of MPO- and CML-stained glomeruli are shown for the sham group (Panel A and D, respectively), the hemodilution group (Panel B and E, respectively) and the CPB group (Panel C and F, respectively). Quantification of the glomerular MPO and CML staining are presented in panel G and H, respectively. *P<0.05 and ***P<0.001 between groups, as tested with Bonferroni post-hoc tests.

Discussion

In this study we questioned whether hemodilution is explanatory for impaired microcirculatory perfusion following cardiopulmonary bypass. In a rat model for extracorporeal circulation we showed that cardiopulmonary bypass led to more microcirculatory disturbances, which were paralleled by increased endothelial activation and increased markers for renal damage, than hemodilution alone. Our findings suggest that the damage of the microcirculation as observed during and after cardiopulmonary bypass can only partially be explained by a dilutional component, but involves a cumulative effect of hemodilution with activation of inflammatory pathways.

The mechanistic cause of impaired microcirculatory perfusion during cardiopulmonary bypass has not yet been elucidated. First, it was assumed that microcirculatory perfusion disturbances originate from systemic hemodynamic alterations, but several strategies with vasoactive medications have failed to preserve perfusion of the microvasculature [28-30]. In the current study, minor decreases in mean arterial pressure were observed after both hemodilution and onset of CPB, whereas particularly in the CPB group, the reduction in microcirculatory perfusion was substantial and did not recover after normalization of mean arterial pressure. Moreover, several studies have demonstrated that raising the mean arterial pressure target to 80 mmHg does not improve clinical outcome or splanchnic function [31,32]. Another cause of microcirculatory impairment may be nonpulsatile flow during CPB. Our group previously showed that pulsatile flow during cardiopulmonary bypass, which is associated with improved endothelial function [33], improved the recovery of microcirculatory perfusion after cardiac surgery [1]. A reduction of intraoperative hemodilution showed similar benefits [34], but neither intervention could prevent the initial decrease in microcirculatory patency. Increased organ inflammatory cytokine expression and endothelial adhesion molecule expression activation induced by CPB [35], as we observed in the CPB group, are known to trigger processes that interfere with microcirculatory perfusion [36]. These processes include increased adhesion of erythrocytes [37], and leukocytes [38], activation of the coagulation system, and endothelial swelling [39]. Our findings demonstrate that the negative impact of cardiopulmonary bypass on microcirculatory perfusion and renal injury is more extensive than that of hemodilution alone. Therefore, other strategies to reduce inflammatory activation, cytokine and endothelial adhesion molecule expression during cardiopulmonary bypass should be investigated in order to preserve the microcirculation and reduce organ dysfunction.

In line with previous studies, hemodilution to a hematocrit of 24% led to decreased microcirculatory perfusion [13] and increased expression of inflammation and endothelial activation [14]. Hemodilution may lead to a reduction in the number of perfused capillaries through the Fahraeus and phase separation effects in the microcirculation [40]. Alternatively,

reduced blood viscosity, independently from the hematocrit level, may impair capillary perfusion during hemodilution, possibly because of reduced endothelial shear stress levels and reduced intracapillary pressure [41]. Finally, reduced erythrocyte deformability during hemodilution may interfere with perfusion of capillaries with low diameters [42]. The current study shows that hemodilution per se is already associated with renal endothelial inflammation when compared to the sham group. From clinical studies, it is known that hemodilution during cardiopulmonary bypass might be a risk factor for acute kidney injury [43], probably through reduced tissue oxygenation following the drop in hematocrit [44]. Although the use of hydroxyethyl starches is controversial nowadays with respect to kidney injury, recent experimental evidence shows that hemodilution with colloids leads to less renal injury than crystalloid hemodilution [15]. As hematocrit correction with perioperative blood transfusion is by itself an independent risk factor for renal failure after cardiac surgery, it is of importance that the associated microcirculatory disturbances due to hemodilution are minimized to reduce additional defects in tissue oxygenation [8].

Albeit no causal relationship, microcirculatory impairment in the rats subjected to CPB was paralleled by inflammatory and endothelial activation and an increased expression of markers for renal injury, while these observations were completely absent in the sham group. It has been established that cardiac surgery associated acute kidney injury is more prevalent following on-pump than off-pump coronary artery bypass grafting [45]. It is likely that the observed microcirculatory disturbances are an important contributor to acute kidney injury observed following cardiac surgery [7,8]. Our findings warrant further exploration of the association between endothelial injury and microcirculatory dysfunction and the consequences for renal function following CPB.

Our study has several limitations. First, although the current rat cardiopulmonary bypass model is a modification of a previously described protocol [20] and enables direct in vivo microcirculatory observations, cardioplegic arrest was not induced in the current study. However, residual flow through the heart was only minimal as there was abolished arterial pulse pressure during CPB. Secondly, the current study is limited by the use of a closed-chest CPB with the accompanying reduction of surgical trauma as compared to the clinical situation. Our results however show that the microcirculatory perfusion alterations [1,11] and endothelial activation and inflammation [46], are well in concordance with results from clinical investigations. Additionally, confounding influence of heparin and protamine on microcirculatory perfusion and endothelial and inflammatory activation were eliminated by equal administration of both drugs in all groups [35]. Since intravital microscopy during CPB in a small animal model has not been described previously, the current setup may help to elucidate the mechanisms behind microcirculatory disturbances during CPB and its consequences for organ dysfunction.

In conclusion, the decrease in microcirculatory perfusion observed during cardiopulmonary bypass can only partly be attributed to hemodilution. Instead, increased cytokine expression and endothelial adhesion molecule expression during CPB are likely more important contributors to the deterioration of the microcirculation. We further showed that impairment of microvascular perfusion is associated with increased markers for renal damage, which is supportive for the hypothesis that microcirculatory perfusion impairment might contribute to the development of postoperative acute kidney injury [7,8]. However, as these hypotheses are based on associative data, further studies are warranted that proof the direct relationship between microcirculatory perfusion disturbances and postoperative renal dysfunction. Our findings furthermore suggest that interventions aimed at the preservation of capillary patency during CPB in order to improve postoperative organ failure should target cytokine expression and endothelial adhesion molecule expression rather than prevention of hemodilution.

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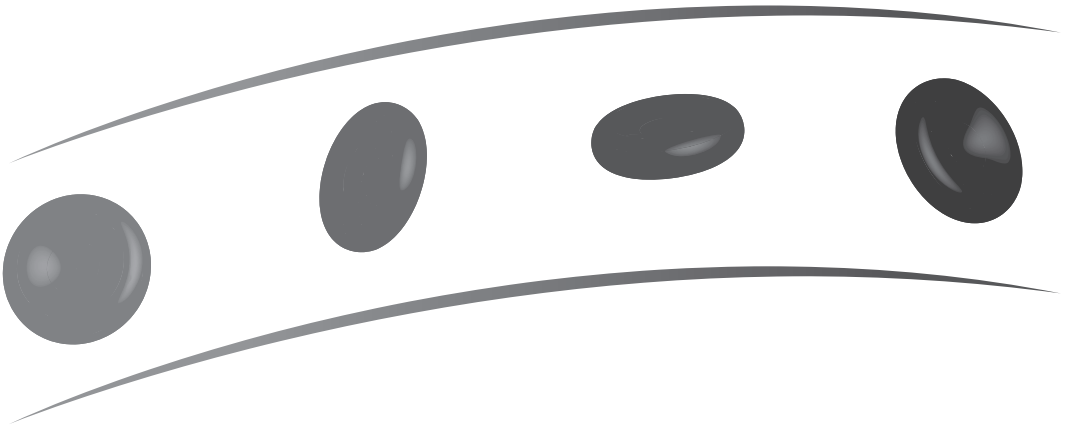

CHAPTER 9

Reduction of vascular leakage by imatinib is associated with preserved microcirculatory perfusion and reduced renal injury in a rat model of cardiopulmonary bypass

N.J. Koning, F. de Lange, M. van Meurs, R.M. Jongman, Y. Ahmed, L.A. Schwarte, G.P. van Nieuw Amerongen, A.B.A. Vonk, H.W.M. Niessen, C. Baufreton, C. Boer.

In submission

Abstract

Background: Cardiopulmonary bypass (CPB) during cardiac surgery leads to impaired microcirculatory perfusion. In the present study we hypothesized that vascular leakage is an important contributor to this perfusion disorder. Using imatinib, acknowledged for its reversal of endothelial barrier dysfunction, we investigated whether prevention of vascular leakage preserves microcirculatory perfusion in a rat model for CPB.

Methods: Anesthetized male Wistar rats were mechanically ventilated, and subjected to 75 minutes of CPB after treatment with imatinib or vehicle (n=8 per group). Cremaster microcirculatory perfusion was measured at six time points before, during and after CPB. Quadriceps microvascular oxygen saturation was measured with reflectance spectrophotometry. Evans Blue Dye (EBD) extravasation was determined in separate experiments and capillary endothelial architecture was assessed with electron microscopy. Inflammatory and endothelial activation and organ injury markers were determined in intestinal, renal and pulmonary tissue.

Results: Onset of CPB decreased the number of perfused microvessels by 40% (9.4 [8.7-10.6] to 5.7 [5.0-6.2] per microscope field, $P<0.001$ versus baseline), whereas this reduction was absent in the imatinib group. In the untreated group, the number of perfused microvessels remained decreased throughout the experiment, while perfusion remained normal in the imatinib group. Microvascular oxygen saturation was less impaired following imatinib treatment compared to controls (6% vs 24% relative reduction during CPB; P=0.030). Imatinib reduced intestinal and pulmonary EBD extravasation and decreased fluid resuscitation volume as compared to control (3.0 [3.0-4.5] vs 12.0 [8.5-14.5] ml; P=0.012). Plasma neutrophil gelatinase-associated lipocalin levels were reduced by imatinib (Control: 1538 [1028-1636] vs Imatinib: 997 [620-1388] ng/ml; P=0.028). Imatinib additionally inhibited ultrastructural signs of endothelial cell injury.

Conclusion: Prevention of endothelial barrier dysfunction using imatinib during CPB attenuated microcirculatory perfusion and oxygenation impairment, reduced fluid resuscitation requirements and reduced of renal and pulmonary injury. Imatinib-induced protection of endothelial barrier integrity and consequent preservation of microcirculatory perfusion and organ function may be considered as novel treatment modality in cardiac surgery.

Introduction

Microcirculatory perfusion is disturbed during cardiac surgery with cardiopulmonary bypass (CPB) in patients and in experimental studies [1-3], but the underlying mechanisms remain to be elucidated. We recently showed that while acute hemodilution during CPB is only a minor and temporary contributor to disturbances of microcirculatory perfusion, inflammation and endothelial activation are more important factors [3]. In particular, CPB was associated with increased markers for renal injury and renal and pulmonary endothelial adhesion molecule expression as compared to similar hemodilution without extracorporeal circulation [3].

Clinical studies and in vitro experiments using endothelial cells in culture show that endothelial activation and inflammation as occurs during CPB elicits endothelial barrier dysfunction with subsequent vascular leakage [4-6]. Moreover, vascular leakage is associated with postoperative pulmonary and renal dysfunction [4,5], which may be mediated by a reduction in capillary perfusion following interstitial fluid accumulation as shown in a model of skeletal muscle ischemia-reperfusion [7]. A reduction of capillary density subsequently reduces tissue oxygenation in line with the Krogh model of oxygen diffusion [8-10]. While the association of vascular leakage with impaired microcirculatory perfusion seems obvious, the number of in vivo studies that support this concept are highly limited. The only available study showed that platelet endothelial cell adhesion molecule-1 (PECAM-1) inhibition reduced endothelial barrier dysfunction and preserved capillary perfusion in parallel in a rat cremaster muscle ischemia-reperfusion model [11].

Imatinib, an inhibitor of multiple tyrosine kinases including c-KIT, platelet-derived growth factor receptor (PDGF-R), c-Abl and Abl-related gene (Arg) [12], prevents endothelial barrier dysfunction after inflammatory stimulation of an endothelial monolayer and reduces vascular leakage in animal models of sepsis and acute lung injury [13,14]. Reduced vascular leakage was attributed to blockade of the c-Abl and Arg pathways [13,14].

These previous findings led to the hypothesis that vascular leakage is an important contributor to impaired microcirculatory perfusion during CPB and that prevention of vascular leakage by imatinib results in preservation of microvascular perfusion and oxygenation by protection of endothelial integrity and barrier function. We additionally investigated whether the preservation of endothelial barrier function reduces organ injury and diminishes the consequences of systemic inflammation induced by cardiopulmonary bypass.

Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the VU University, and were conducted following the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the Dutch Animal Experimentation Act. Animals were housed four per cage with unrestricted access to food and water in a temperature-controlled room (20–23°C; 40–60% humidity) with a 12/12 h light dark cycle. Animal care was performed according to the national guidelines for care of laboratory animals. A total of 24 male Wistar rats of 375–425 g (Charles River Laboratories, Brussels, Belgium) were included to undergo CPB procedures. Sixteen rats were randomized to treatment with imatinib (CPB imatinib; n=8) or saline (CPB control; n=8) before undergoing cardiopulmonary bypass. All measurements were performed in these groups, except for vascular leakage quantification. Vascular leakage was determined in separate experiments, in which eight additional rats were randomized to undergo cardiopulmonary bypass with (n=4) or without imatinib (n=4).

Anesthesia and surgical preparation

Anesthesia was induced as previously described (3). Briefly, anesthesia induction with 5% isoflurane in oxygen followed by endotracheal intubation with a 16G catheter (Venflon Pro, Becton Dickinson, Helsingborg, Sweden). Mechanical ventilation (UMV-03, UNO Roestvaststaal BV, Zevenaar, The Netherlands; tidal volume 10ml/kg, frequency 60-65/min, PEEP 2-4 cmH2O) was initiated and anesthesia was maintained with 2-3% isoflurane with the FiO_2 at 0.35. Body temperature before and after CPB was maintained at 36.5 °C.

A 22G catheter (Venflon Pro, Becton Dickinson, Helsingborg, Sweden) was inserted in the tail artery for arterial blood pressure monitoring. Fentanyl (12 µg/kg, Janssen-Cilag, Tilburg, the Netherlands) was then administered and repeated before onset of CPB and after 40 minutes of CPB. Inhaled isoflurane concentration was reduced to 1.5-2.0%. Left cremaster muscle isolation occurred under continuous warm saline superfusion according to the technique previously described by Baez [15], and the exposed muscle was covered with gas impermeable plastic film (Saran Wrap, Dow Chemical, Midland, USA; presoaked for 24 hours in distilled water). The left quadriceps muscle was then exposed to the fascia under superfusion of warm saline and covered with gas impermeable plastic film. A first dose of heparin (500 IU/kg, LEO Pharma, Amsterdam, The Netherlands) was administered. The arterial inflow cannula (20G Arterial Cannula, Becton Dickinson, Helsingborg, Sweden) of the CPB system was placed in the right femoral artery. At 30 minutes before onset of CPB, rats received an intraperitoneal injection with imatinib mesylate (SelleckChem, Houston, USA) 50 mg/kg, 25 mg/ml in 0.9% sodium chloride (imatinib group) or an equal volume of 0.9% sodium chloride

in the control group [13]. Before onset of cardiopulmonary bypass, a second dose of heparin (500 IU/kg) and pancuronium bromide (0.5 mg/kg, Organon, Oss, the Netherlands) were administered. The right jugular vein was cannulated with a modified multi-orifice 4.5 French catheter (Desilets-Hoffman, Cook, Bloomington, IN, USA) that was placed with the tip in the right atrium, after which extracorporeal circulation was started. Arterial blood gas analysis (Radiometer ABL-50, Radiometer, Brønshøj, Denmark) and hematocrit measurement were performed at baseline and at 10, 30 and 60 minutes of extracorporeal circulation and 10 and 60 minutes after discontinuation of CPB. After the final microcirculatory recordings, plasma was sampled, rats were sacrificed and renal, pulmonary and intestinal tissues were harvested.

Rat cardiopulmonary bypass protocol

The CPB circuit consisted of a Plexiglas venous reservoir, a roller pump (Pericor SF70, Verder, Haan, Germany), and a 4 ml Plexiglas oxygenator-heat exchanger (Ing. M. Humbs, Valley, Germany) with a three layer hollow fiber membrane (Oxyphan, Membrana, Wuppertal, Germany) for gas exchange [16]. A 1.0 mm diameter arterial line (LectroCath, Vygon, Ecouen, France) was connected to the femoral artery cannula. The circuit was primed with 10 ml of 6% hydroxyethyl starch (HES; Voluven 130/0.4, Fresenius Kabi, Halden, Norway). Flow rates of 150-200 ml/kg/min were maintained during cardiopulmonary bypass, equivalent to 100% of the normal rat cardiac output [17]. Venous cannula repositioning was used to minimize residual blood flow through the heart if necessary, based on the drainage rate and minimized residual arterial pulsations. Mechanical ventilation was discontinued, a mixture of oxygen and carbon dioxide was led through the oxygenator membrane to maintain pO_2 values between 150 and 200 mmHg and pCO_2 levels between 32 and 45 mmHg. Isoflurane (1.0-1.5%) was added to the gas mixture. Mild hypothermia was applied during CPB at 35.0°C. At 60 minutes of CPB, rats were slowly rewarmed to 36.0-36.5°C. Mechanical ventilation was restarted 10 minutes before discontinuation of CPB. Disconnection from CPB occurred after 75 minutes of extracorporeal circulation, the venous cannula was removed and the jugular vein was clamped. Protamine hydrochloride (2 mg/kg) was administered to neutralize heparin 15 minutes after weaning from CPB.

Microcirculatory measurements

Intravital microcirculatory observation was performed on the cremaster muscle, which has a microvascular bed that maintains capillary patency throughout the duration of the experiment in sham conditions [3]. A 10x objective (WN-Achroplan, Zeiss, Oberkochen, Germany; numerical aperture 0.30) on an intravital microscope (AxiotechVario 100HD, Zeiss) was connected to digital camera (scA640, Basler, Ahrensburg, Germany). The final magnification was 640x. At baseline, 3 regions of undisturbed tissue architecture and normal perfusion were selected for measurements. In each region, four videos of 10 to 15 seconds were recorded

at each time point, combining for twelve videos per time point. Baseline measurements were performed after a 30-minute stabilization period of the exposed cremaster muscle. Subsequent measurements were made at 10, 30 and 60 minutes of CPB and 10 and 60 minutes after discontinuation of CPB. Microcirculatory perfusion analyses were performed off-line and the investigator was blinded for the allocated group. As previously described, capillary crossings with two vertical test lines were counted per screen and subdivided in perfused, intermittently perfused and nonperfused capillaries [3,9].

Microvascular hemoglobin oxygen saturation ($\mu HbSO_2$) in the microcirculation was measured with reflectance spectroscopy (O2C, LEA Medizintechnik GmbH, Giessen, Germany) on the left quadriceps muscle [18]. White light (450-1000 nm) was illuminated by a microprobe and reflected light was analyzed for the absorption spectra of oxygenated and deoxygenated hemoglobin in the tissue. As vessels with a diameter larger than 100 µm completely absorb the emitted light [19], $\mu HbSO_2$ results reflect the hemoglobin oxygenation in smaller vessels, mainly in post-capillary venules.

Vascular leakage

Evans Blue dye extravasation was used for the analysis of vascular leakage as previously described [13]. In a separate series of experiments, 8 rats underwent cardiopulmonary bypass with (n=4) or without (n=4) treatment with imatinib (50 mg/kg i.p.). Evans Blue dye (Merck, Darmstadt, Germany; 2.5 ml/kg i.v. of 10 mg/ml in 0.9% sodium chloride) was administered after discontinuation of cardiopulmonary bypass. After 60 minutes, the animals were perfused with 200 ml 0.9% sodium chloride in order to remove the intravascular Evans Blue dye. Intestinal, pulmonary and renal tissues were harvested and weighed and incubated in 200 µl (intestine) or 600 µl (lung and kidney) formamide (Sigma-Aldrich, St. Louis, USA) at 55°C for Evans Blue dye extraction. After 48 hours, organs were washed and dried at 90° Celsius. Formamide was centrifuged for 5 minutes at 13500 rpm and analyzed with spectrophotometry for absorbance at 610 nm for Evans Blue Dye and 740 nm for remaining heme pigments. Evans Blue absorbance was calculated with: $OD_{610} - [1.426 * OD_{740} + 0.03]$ and subsequently corrected for organ dry weight [13].

Immunohistochemical analyses

Immunohistochemical analysis of renal paraffin sections stained with myeloperoxidase (MPO) and N(epsilon)-(carboxymethyl)lysine (CML) was performed as previously described [3,20], in order to assess glomerular neutrophil infiltration and inflammatory status of the glomerular endothelium, respectively. As primary antibodies, mouse-anti-rat MPO (1:50, Abcam, Cambridge, UK) or CML (1:2000) were used (3,49). In anti-MPO stained sections, the number of neutrophils was determined in 100 glomeruli per section. In CML-stained sections, a semi-quantitative scoring system ranging from 0 (no positivity) to 3 (strong positivity) was used

[21]. An average of 100 glomeruli was used as intensity score. The investigator performing the analysis was blinded for group allocation.

Gene expression analysis

Total RNA isolation from cryosections from renal, pulmonary and intestinal tissue occurred as previously described [3], using the RNeasy Mini Plus Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. Gel electrophoresis was used to determine integrity of RNA was determined. RNA yield and purity were measured with NanoDrop®ND-1000 UVvis spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA was reverse-transcribed in cDNA using SuperScript®III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). The following Assays-on-Demand primers (Applied Biosystems Systems, Foster City, CA) were used for PCR: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; assay ID Rn01775763_g1), E-selectin (assay ID Rn00594072_m1), P-selectin (assay ID Rn00565416_m1), intercellular adhesion molecule-1 (ICAM-1; assay ID Rn00564227_m1), vascular cell adhesion molecule-1 (VCAM-1; assay ID Rn00563627_m1), tumor necrosis factor-α (TNF-α; assay ID Rn00562055_m1), interleukin-6 (IL-6; assay ID Rn00561420_m1), interleukin-10 (IL-10; assay ID Rn00563409_m1), monocyte chemoattractant protein-1 (MCP-1; assay ID Rn00580555_m1) and MPO (assay ID Rn00585552_m1). Samples were run in duplicate and the obtained threshold cycle values (CT) were averaged. Gene expression was normalized to the expression of the housekeeping gene GAPDH (ΔCT). mRNA levels were calculated by 2-ΔCT values and per group averaged.

Preparation of tissue homogenates

Cryo sections from renal, pulmonary and intestinal tissue were cut and lysed on ice in RIPA buffer (50mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) IGEPAL) containing protease inhibitor cocktail (Roche Diagnostics, Almere, the Netherlands), phosphatase inhibitor (Roche Diagnostics) and activated 1mM Na_3VO_4. Homogenates were sonicated two times for 5 seconds on ice and centrifuged for 10 minutes at 14000g at 4°C. Total protein concentration in the supernatant were determined using a DC Protein Assay (Bio-Rad Laboratories, Hercules, USA).

Protein expression in tissue homogenates and plasma

Protein levels in tissue homogenates of TNF-α (438207, BioLegend, London, UK), MCP-1 (MJE00, R&D Systems, Abingdon, UK), IL-1β (RBL00, R&D Systems) and neutrophil gelatinase-associated lipocalin (NGAL; KIT 046, BioPorto, Hellerup, Denmark) and plasma levels of TNF-α, MCP-1, IL-6 (437107, BioLegend), NGAL and MPO (HK105, Hycult Biotech, Uden, the Netherlands) were measured by ELISA in plasma and in tissue homogenates according the

manufacturer's instructions. Obtained protein levels for tissue homogenates were corrected for the total protein input of tissue homogenate and expressed as pg protein of interest per µg total protein content.

Electron microscopy

Intestinal tissue was used for analysis of capillary endothelial ultrastructure, given the association of intestinal endothelial barrier dysfunction with the development of organ dysfunction [22,23]. After fixation in 4% buffered formaldehyde, intestinal tissue was post fixed in 1% osmium tetroxide. The tissue was then dehydrated through a graded series of ethanol resolutions of 70-95% and embedded in JB-4 Plus resin. Thereafter, 0.5-3 µm thick sections were cut with a glass knife. These semithin sections were processed for electron microscopy. A JEOL-1200 EX (JEOL, Tokyo, Japan) electron microscope was used. Ten random capillaries captured in full cross-section by an investigator blinded for group allocation were then analyzed for signs of endothelial cell injury, including the number of luminal membrane blebs and vacuoles [24-26]. Additionally, breaches in the luminal barrier formed by the endothelial cells were counted.

Statistical analysis

Data were analyzed using a SPSS statistical software package (IBM, version 17.0). All values are expressed as median with interquartile range [IQR]. Sample size was calculated for capillary perfusion as primary outcome and was based on a previous study in which nonpulsatile cardiopulmonary bypass induced a maximal decrease in sublingual perfused capillaries from 18.9 ± 3.5 mm/mm^2 to 13.2 mm/mm^2 in humans [2]. Using an alpha of 0.05 and a power of 0.9, a sample size of 8 per group was required. Time-dependent changes between groups were analyzed using ANOVA repeated measures. Two-sided Mann-Whitney U and Wilcoxon tests were used to evaluate differences in between groups and within groups, respectively. Post-hoc Bonferroni correction was applied for parameters with repeated measurements. $P < 0.05$ was considered as statistically significant.

Results

All animals completed the experimental protocol and were analyzed on an intention to treat basis. Rats weighed 410 g [398-425] and 403 g [399-410] for CPB control and CPB imatinib groups, respectively (P=0.315).

Systemic variables

Imatinib did not alter mean arterial pressure at baseline, while mean arterial pressure decreased in both groups during CPB, and augmented after disconnection from CPB (Figure 1A). Hematocrit levels decreased by 48% (P<0.001 versus baseline) in the control group and by 52% (P<0.001 versus baseline) in the imatinib group after onset of CPB (Figure 1B). There were no differences between groups in either blood pressure or hematocrit values during the course of the experiments.

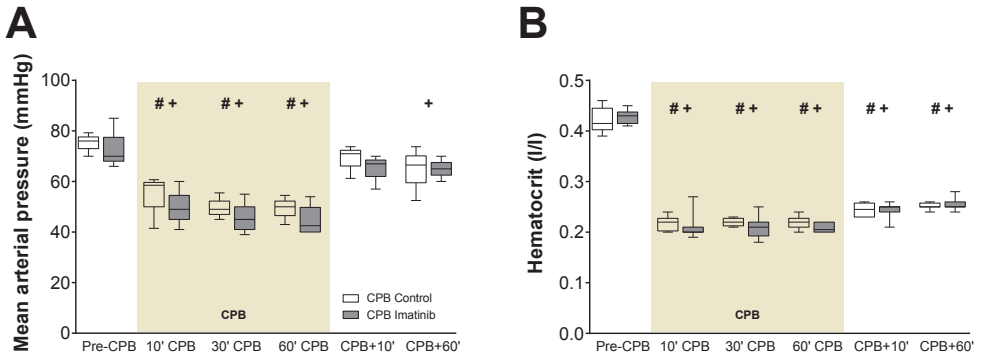

Figure 1 Levels of mean arterial blood pressure (Panel A) and hematocrit (Panel B) during the experiments for the control group (white) and the imatinib group (grey). The grey background indicates the period during cardiopulmonary bypass. No between-group differences were detected. #P<0.05 Control group vs baseline, +P<0.05 Imatinib group vs baseline as tested with a Wilcoxon test.

In vivo microcirculatory perfusion and oxygenation

The number of perfused capillaries in the cremaster muscle at baseline was similar between groups. In the control group, the number of perfused capillaries decreased by 39% (P<0.001) after onset of CPB (Figure 2A). In rats treated with imatinib, a non-significant temporary reduction of 7% in the number of perfused capillaries was observed (P=0.054). At all time points during and after CPB, the number of perfused capillaries was higher after imatinib treatment than in the control group (ANOVA RM P=0.007). Imatinib did not alter the number of nonperfused vessels at baseline. Both groups showed an increase in nonperfused vessels 10 minutes after onset of CPB (Control: 0.1 [0.0-0.5] to 1.1 [0.7-1.8] per microscope screen; P=0.012; Imatinib: 0.3 [0.2-0.4] to 0.7 [0.4-1.3] per microscope screen; P=0.012). However,

this increase in nonperfused vessels as compared to baseline was no longer detected in the subsequent time points in the imatinib group, whereas this elevation persisted in the control group throughout the experiment (Figure 2B). Similar to microcirculatory perfusion, microcirculatory hemoglobin oxygen saturation is better preserved during CPB with imatinib treatment (Figure 2C).

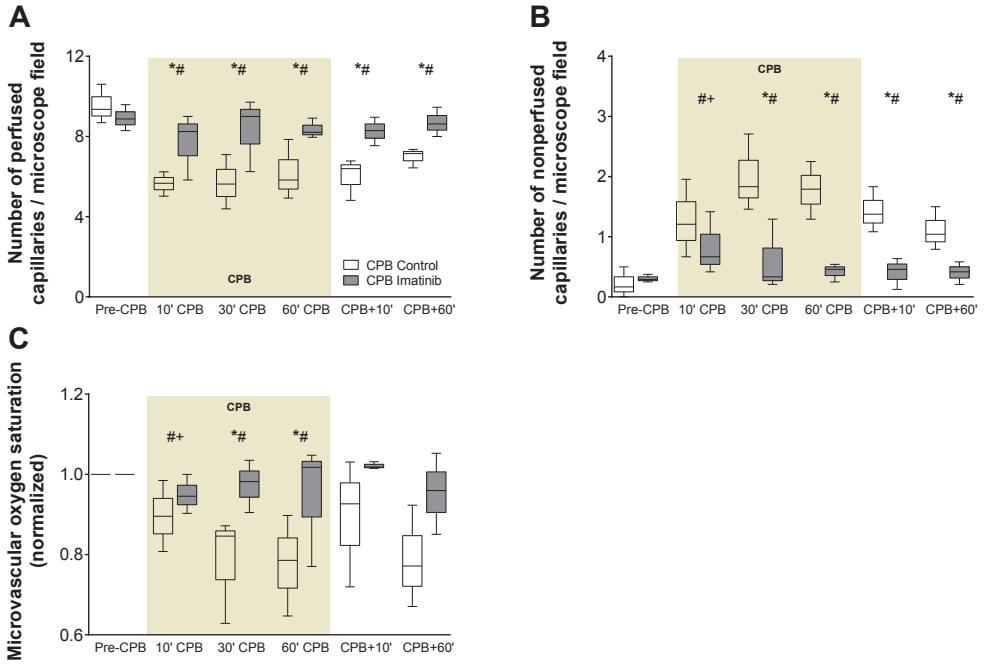

Figure 2 Number of perfused (Panel A) and nonperfused capillaries (Panel B) assessed with cremaster muscle intravital microscopy and microvascular hemoglobin oxygen saturation in the quadriceps muscle (Panel C) for the control group (white) and the imatinib group (grey). *P<0.05 Control vs Imatinib as tested with a Mann-Whitney U test, #P<0.05 Control group vs baseline, +P<0.05 Imatinib group vs baseline as tested with a Wilcoxon test.

The control group had a $\mu HbSO_2$ of 0.85 [0.63-0.87] and 0.79 [0.65-0.90] of baseline at 30 and 60 minutes of CPB respectively, whereas this was 0.98 [0.90-1.04] and 1.02 [0.77-1.05] of baseline for the imatinib group (P=0.11 and P=0.028 between groups at 30 and 60 minutes of CPB, respectively).

Vascular leakage

Vascular leakage rates in the hour after CPB with and without imatinib treatment are presented in Figure 3 for renal (Panel A), pulmonary (Panel B) and intestinal tissue (Panel C). Intestinal and pulmonary extravasation following CPB was reduced with imatinib as compared to the

control group, whereas no significant difference in renal vascular leakage between groups was detected.

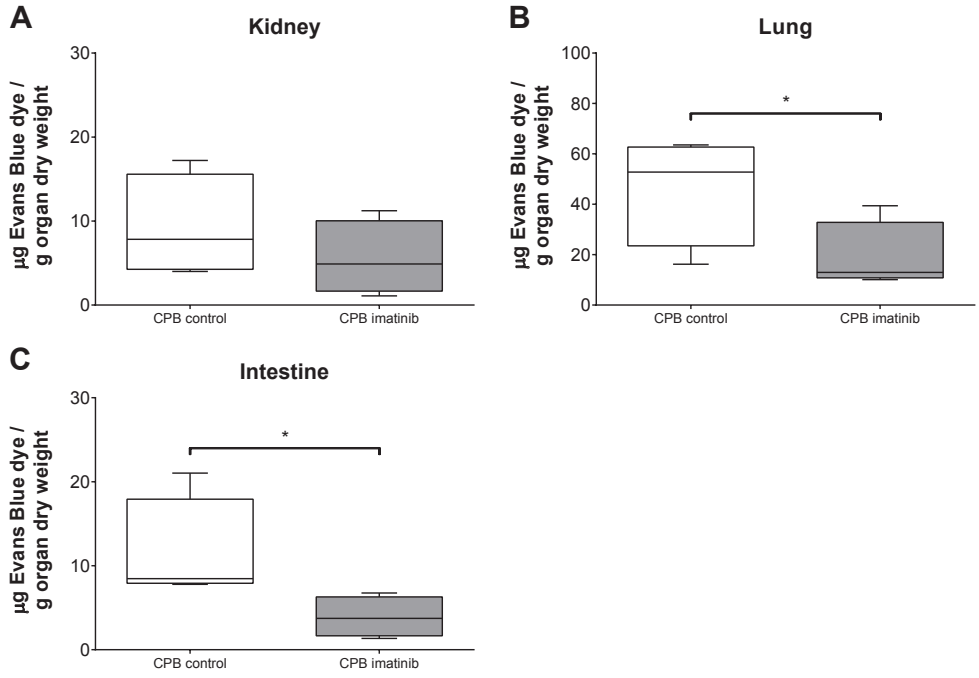

Figure 3 Vascular leakage following disconnection from cardiopulmonary bypass as assessed by Evans Blue Dye extravasation corrected for organ dry weight in the control group (white; n=4) and the imatinib group (grey; n=4). *P<0.05 between groups as tested with a Mann-Whitney U test.

Imatinib treatment reduced the need for fluid resuscitation during and after CPB by 75% compared to the control group (Imatinib: 3.0 [3.0-4.5] ml vs Control: 12.0 [8.5-14.5] ml; P=0.024).

Renal immunohistology

Imatinib treatment significantly reduced glomerular neutrophil infiltration as compared to the control group (Figure 4A). Moreover, glomerular endothelial CML intensity was significantly lower in the imatinib group than in the control group, demonstrating that imatinib treatment leads to a reduction of endothelial inflammatory status after CPB (Figure 4D).

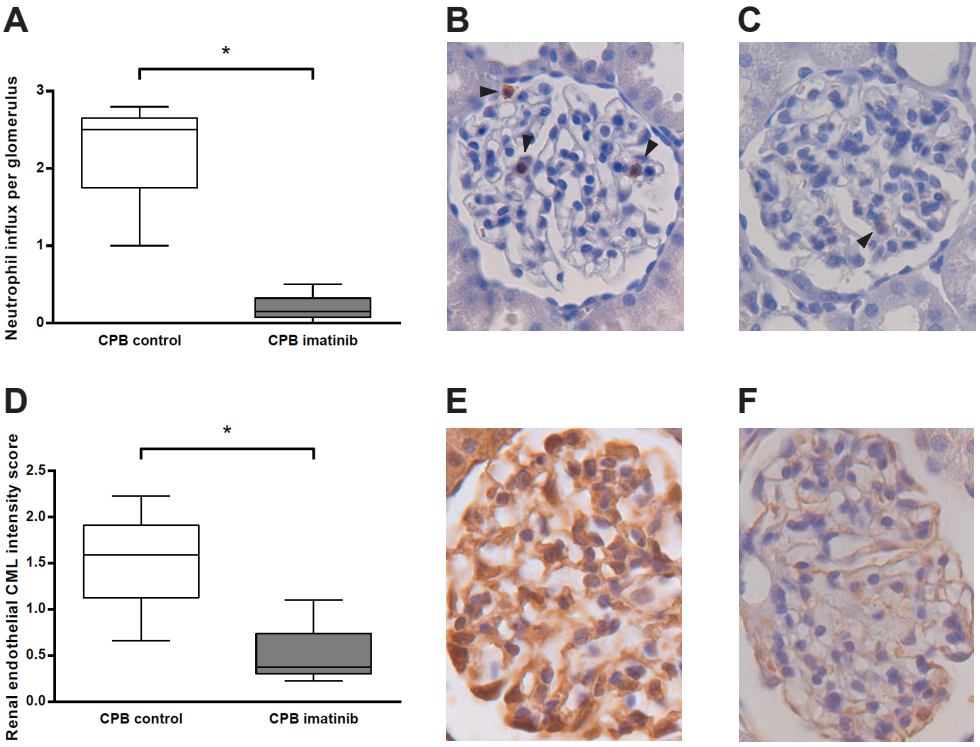

Figure 4 Glomerular neutrophil influx (Panel A) as assessed by anti-MPO staining for the control group (white) and the imatinib group (grey) and typical examples of the control group (Panel B) and the imatinib group (Panel C), neutrophils are indicated by black arrows. Renal glomerular endothelial inflammatory status (Panel D) as assessed by CML staining with typical examples of the control group (Panel E) and the imatinib group (Panel F). *P<0.05 between groups as tested with a Mann-Whitney U test.

Inflammatory cytokine and endothelial activation

Imatinib treatment decreased pulmonary inflammation as demonstrated by reduced levels of pulmonary IL-1β, IL-6, TNF-α and MCP-1 mRNA expression (Figure 5) and pulmonary IL-1β, TNF-α and MCP-1 protein expression (Figure 6) after CPB as compared to the control group.

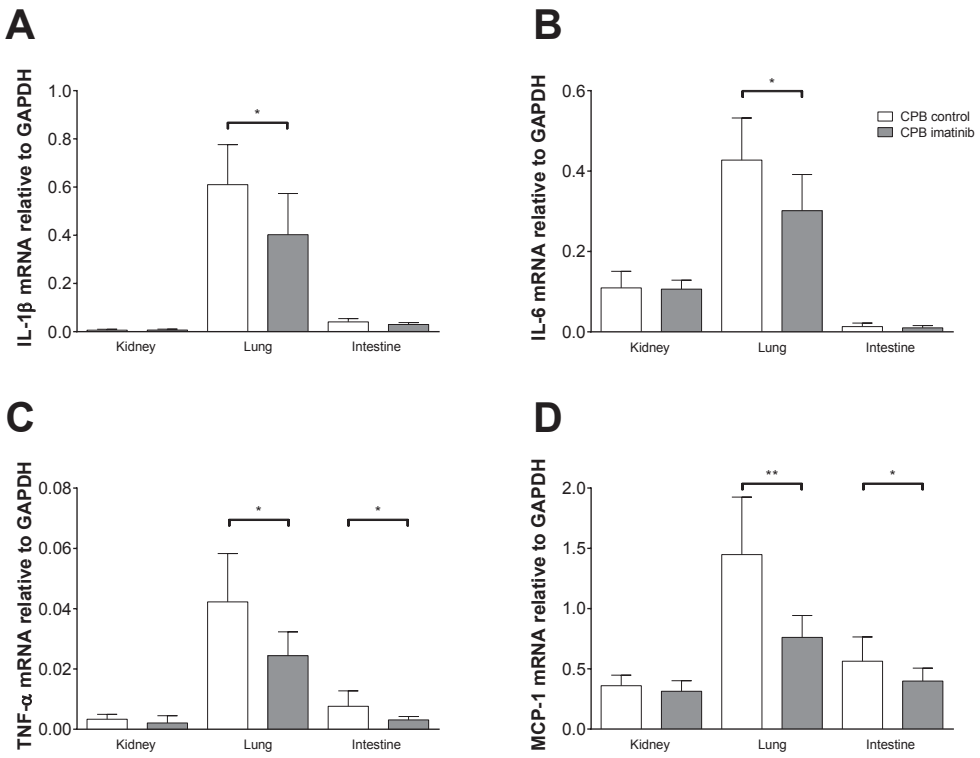

Figure 5 Renal, pulmonary and intestinal mRNA expression of IL-1β (Panel A), IL-6 (Panel B), TNF-α (Panel C) and MCP-1 (Panel D) for the control group (white bars) and the imatinib group (grey bars). *P<0.05 and **P<0.01 between groups as tested with a Mann-Whitney U test.

Similarly, intestinal MCP-1 mRNA expression and protein levels and TNF-α mRNA expression were reduced by imatinib treatment. Plasma levels of MCP-1 were reduced by imatinib treatment as compared to control (32 [22-39] vs 46 [40-65] ng/ml, respectively; P=0.005) whereas a nonsignificant decrease in the plasma levels of IL-6 (Imatinib: 58 [44-88] vs Control: 121 [85–151] ng/ml; P=0.088]) and TNF-α (Imatinib: 1.1 [0.7–8.3] vs Control: 3.8 [0.9-12.5] ng/ml; P=0.232]) was observed following imatinib treatment when compared to control.

Imatinib treatment led to reduced pulmonary P-selectin mRNA expression after CPB (Control: 3.2 [2.6-4.7] vs Imatinib: 2.2 [1.8-2.8] relative to GADPH, P=0.036; Figure 7). For the late endothelial adhesion molecules VCAM-1 and ICAM-1, no differences in pulmonary mRNA expression could be detected between groups. No differences in endothelial adhesion molecules were observed in intestinal or renal tissue.

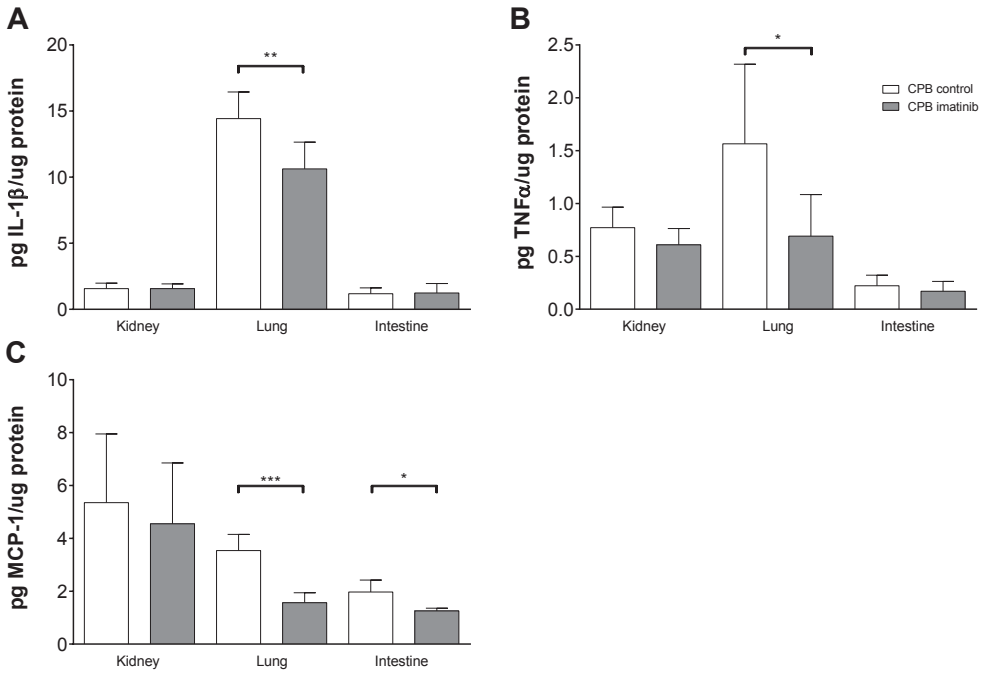

Figure 6 Renal, pulmonary and intestinal IL-1β levels (Panel A), TNF-α levels (Panel B) and MCP-1 levels (Panel C) for the control group (white bars) and the imatinib group (grey bars). *P<0.05, **P<0.01 and ***P<0.001 between groups as tested with a Mann-Whitney U test.

Organ injury

Imatinib treatment reduced plasma NGAL levels (Control: 1538 [1028-1636] vs Imatinib: 997 [620-1388] ng/ml; P=0.028) and tended to decrease in renal NGAL protein levels (Control: 164 [111-244]; Imatinib: 138 [80-214] pg/µg protein; P=0.317). Treatment with imatinib additionally decreased plasma MPO levels (Control: 11.7 [9.9-17.0] vs Imatinib: 7.8 [6.2-9.2] ng/ml; P=0.002). Finally, imatinib reduced MPO mRNA expression in pulmonary (Control: 1.0·10-3 [0.9-1.6] vs Imatinib: 0.4·10-3 [0.4-0.6] relative to GAPDH; P=0.011) but not renal tissue (Control: 0.07·10-3 [0.04-0.9] vs Imatinib: 0.04·10-3 [0.02-1.1] relative to GAPDH; P=0.516) after CPB, signaling altogether reduced renal and pulmonary injury following CPB with imatinib treatment.

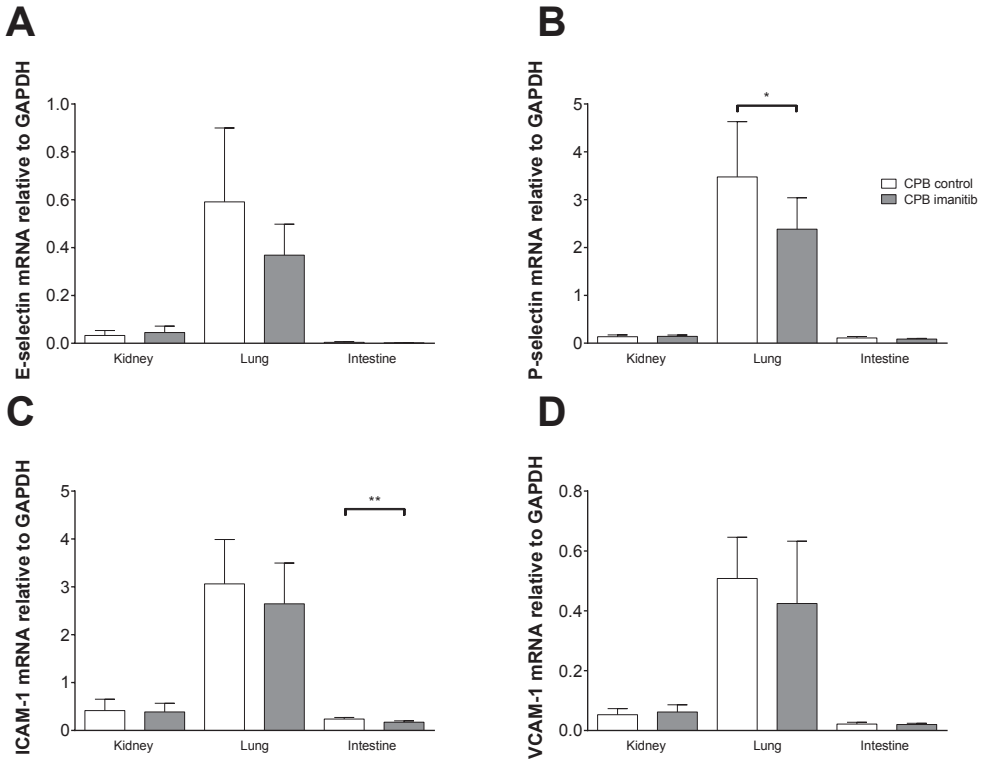

Figure 7 Renal, pulmonary and intestinal mRNA expression of the adhesion molecules E-selectin (Panel A) and P-selectin (Panel B), ICAM-1 (Panel C) and VCAM-1 (Panel D) for the control group (white bars) and the imatinib group (grey bars). *P<0.05 and **P<0.01 between groups as tested with a Mann-Whitney U test.

Endothelial electron microscopy

Intestinal capillary endothelial ultrastructure was better preserved after CPB with imatinib in comparison with the control group (Figure 8). Imatinib reduced the number of endothelial barrier breaches compared with the control group (Control: 2 [1-6] vs Imatinib: 0 [0-2] endothelial breaches per capillary cross-section; P=0.019). Moreover, in the control group, endothelial vacuolization (14 [9-18] vs 9 [6-12] vacuoles per capillary cross-section with imatinib; P=0.034) and endothelial membrane blebbing (24 [13-36] vs 14 [9-19] blebs per capillary cross-section with imatinib; P=0.012) were detected more frequently than in the imatinib group.

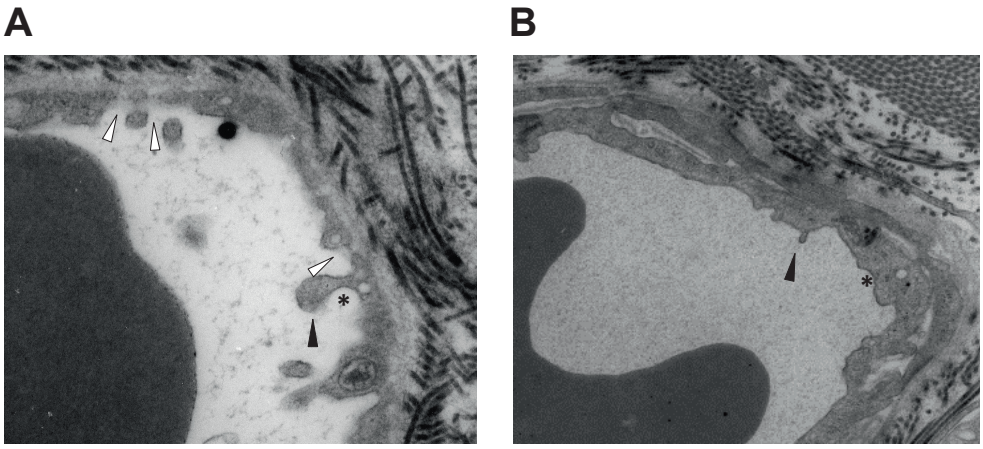

Figure 8 Example of intestinal capillary endothelial ultrastructure analysis with electron microscopy. Breaches in the endothelial barrier (white arrows), membrane blebbing (black arrows) and vacuolization (asterisk) are visible in a cross-section of a capillary from the control group (Panel A) and in the imatinib group (Panel B).

Discussion

Although cardiopulmonary bypass in cardiac surgery is commonly associated with microcirculatory perfusion disturbances, the underlying pathophysiological mechanisms and effects on organ function are hardly known. Our recent studies suggest that the cause for microvascular perfusion disturbances during cardiopulmonary bypass should be sought in the vascular endothelium, in particular dysfunction of the endothelial barrier [6,27,28]. We therefore investigated whether protection of endothelial barrier integrity by imatinib preserves microcirculatory perfusion, with specific emphasis on vascular leakage as common pathway.

Our study demonstrates that a reduction of systemic vascular leakage using imatinib in rats undergoing cardiopulmonary bypass preserves microcirculatory perfusion and oxygenation. This was associated with reduced fluid resuscitation requirements during and after cardiopulmonary bypass. The endothelial cell ultrastructure and endothelial barrier was better preserved in the imatinib group than in the control group. Additionally, we demonstrate that imatinib treatment resulted in a reduction in endothelial activation and inflammation in the kidney, the lung and the gastrointestinal system, paralleled by a decrease in renal and pulmonary injury markers. We are the first to show the important contribution of vascular leakage to the disturbances in microcirculatory perfusion during cardiopulmonary bypass. Our findings are an important step in unrevealing the pathogenesis underlying microcirculatory dysfunction in acute inflammatory activation.

The impairment of microcirculatory perfusion during cardiopulmonary bypass has been studied extensively but the underlying mechanisms have remained unrevealed so far [1,2,29]. Microcirculatory disturbances appeared to occur independent from alterations in macrocirculatory parameters [2]. Treatment with vasoactive medication was unable to augment capillary density during cardiopulmonary bypass [30-32]. We recently showed in our rat model for cardiopulmonary bypass that hemodilution to a hematocrit of 0.24 l/l led to a minor and temporary decrease in perfused capillaries and could not explain the microcirculatory impairment observed during CPB [3]. In contrast, pulsatile flow during cardiopulmonary bypass was associated with improved postoperative microvascular recovery as compared to nonpulsatile flow [2]. Moreover, pulsatile flow reduces inflammation of the vascular wall ex vivo as compared to nonpulsatile flow [33], suggesting a role for mechanotransduction in the preservation of endothelial function and subsequently microcirculatory perfusion. The important role of the vascular wall in the pathogenesis of microvascular perfusion impairment during CPB was confirmed by a later observation that reduced endothelial glycocalyx dimensions were associated with reduced microcirculatory perfusion in patients undergoing cardiac surgery [27].

Increased fluid extravasation interferes with tissue oxygenation at two levels: extravascular compression of capillaries leads to a decrease perfused capillary density [7], and an increased barrier for diffusion of oxygen from the capillary to the cell according to the Krogh model [8-10]. That a disturbance in the balance between intracapillary and interstitial pressures may lead to compromised microvascular perfusion was additionally shown by Cabrales et al. [34]. In their hamster window chamber model, they showed that sufficient intracapillary pressure was required to maintain perfused capillary density during acute normovolemic hemodilution [34]. The current results show that prevention of vascular leakage preserves microcirculatory perfusion during CPB and therefore supports the hypothesized important role for vascular leakage in the development of microvascular perfusion disorders during CPB. Since endothelial barrier dysfunction is present in humans undergoing cardiac surgery with CPB [4-6], this pathophysiological mechanism can be considered as potential therapeutic target for improvement of microcirculatory perfusion and reduction of organ injury by endothelial barrier protection in a clinical situation.

The protection of endothelial barrier function exerted by imatinib was reflected by a reduction in vascular leakage in the lung and the intestines. Moreover, we showed that intestinal capillary endothelial ultrastructure was less disturbed after cardiopulmonary bypass in imatinib treated rats when compared to the control group. In particular, imatinib reduces breaches in the endothelial barrier and consequently the area of direct contact of the capillary lumen with the capillary basement membrane, that facilitates vascular leakage. Interestingly, fluid resuscitation requirements during and after CPB were reduced by 75% following imatinib treatment when compared to untreated animals, while maintaining similar hematocrit levels and mean arterial pressure values throughout the experiment. Previous clinical studies have shown that on-pump cardiac surgery induces endothelial barrier dysfunction [4-6]. In the study of Clajus et al., the mean postoperative 24 hour fluid balance was 11 liters positive, indicating substantial vascular leakage [5]. Fluid overload following cardiac surgery has been found to be an independent predictor of postoperative morbidity [35], and its burden may be reduced by interventions that preserve endothelial barrier function. Our results demonstrate that reduction of vascular leakage using imatinib leads to a more effective intravascular volume expansion by resuscitation fluids, which may substantially diminish the positive perioperative fluid balance in on-pump cardiac surgery.

The association between impaired microcirculatory perfusion and poor clinical outcome has been observed for sepsis, cardiogenic shock and abdominal surgery [36-38], but not for cardiac surgery. The hypothesized important role for microcirculatory perfusion disturbances in the development of acute organ dysfunction as acute kidney injury [39-41] or acute respiratory distress syndrome [42,43], is supported by our findings. Preservation of microcirculatory perfusion was followed by reduced renal glomerular endothelial inflammation and neutrophil deposition and reduced plasma levels of NGAL, a marker of tubular damage and predictor of

acute kidney injury in patients undergoing cardiac surgery [44,45]. Both groups demonstrated NGAL plasma levels that indicate substantial tubular injury [45]. Acute kidney injury following cardiopulmonary bypass remains a multifactorial disease associated with increased mortality [46,47], however so far advances in etiology and therefore in rational pharmacological therapy are missing [41,48]. Our results strongly suggest that preservation of microvascular perfusion during CPB using imatinib is associated with reduced signs of injury and inflammation in both the kidney, the lung, and the intestines.

Imatinib reduced the inflammatory and endothelial activation response in intestinal, pulmonary and renal tissue and in plasma when compared to the control group. Since both groups were exposed to equal inflammatory activation induced by onset of cardiopulmonary bypass [49], the difference in inflammatory activation markers is remarkable. The reduced inflammation and endothelial activation in the imatinib group as compared to the control group may hypothetically be an effect secondary to preserved microvascular perfusion. Imatinib has however been associated with reduced inflammation in previous in vivo experimental investigations [50], although this anti-inflammatory effect may only appear after prolonged exposure [51]. This direct anti-inflammatory effect by imatinib was not investigated in the current study. A treatment combining anti-inflammatory and endothelial barrier protective effects may however be advantageous in on-pump cardiac surgery [52]. Moreover, imatinib is a registered drug for the treatment of chronic myeloid leukemia and extensive experience has already been generated with its use, making it a suitable candidate for further clinical investigation of its possible benefits on microvascular perfusion and organ function in on-pump cardiac surgery.

The current study has several limitations. Our current rat model is a beating heart peripheral cardiopulmonary bypass model with a beating heart. However, it enabled conduct of total cardiopulmonary bypass with minimalized pulse pressure and discontinuation of ventilation during CPB without the occurrence of hypoxemia or cardiac complications. Moreover, all physiologic alterations as occur during human cardiopulmonary bypass, e.g. hemodilution, inflammatory activation, nonpulsatile flow, mild hypothermia are present in the current model, which makes it resemblance to the human setting relatively high [53].

Although the use of biomarkers for renal injury may be controversial, we did not expect creatinine clearance to be a sensitive marker of renal injury within the short time course of our study [54]. We therefore used plasma NGAL levels as a renal injury marker, which had a good predictive value of AKI in adult cardiac surgical patients [55].

The extracorporeal circuit was primed with a colloid solution (HES 130/0.4), which may be controversial, in particular when investigating acute kidney injury. However, it has been shown that acute normovolemic hemodilution using crystalloids is more deleterious for renal function and microvascular oxygenation as compared to colloids [56]. A prospective cohort study confirmed that use of modern HES solutions were not associated with increased rates of AKI in a cardiac surgical population [57].

This study did not investigate by which pathway imatinib exerted its preserving effects on the endothelial barrier function. Multiple in vitro studies have however confirmed the importance of the Abl kinases c-Abl and Arg in the action of imatinib of endothelial barrier dysfunction [13,14,58]. In thrombin-stimulated endothelial cell monolayers, Arg-knockdown had similar effects as imatinib treatment, whereas there were no addictive effects during combined treatment [13].

In conclusion, our findings suggest that vascular leakage plays an important role in the development of impaired microcirculatory perfusion and oxygenation and organ dysfunction following cardiopulmonary bypass. Moreover, reduction of vascular leakage using imatinib treatment decreases microcirculatory alterations and reduces fluid resuscitation requirements, inflammation and organ injury in a rat model of cardiopulmonary bypass. Patients undergoing on-pump cardiac surgery may benefit from a treatment that reduces endothelial barrier dysfunction in order to reduce postoperative organ dysfunction and the consequences of systemic inflammation. Therefore, imatinib administration should be investigated in patients undergoing cardiac surgery with cardiopulmonary bypass in patients at risk of postoperative organ dysfunction due to perfusion disturbances.

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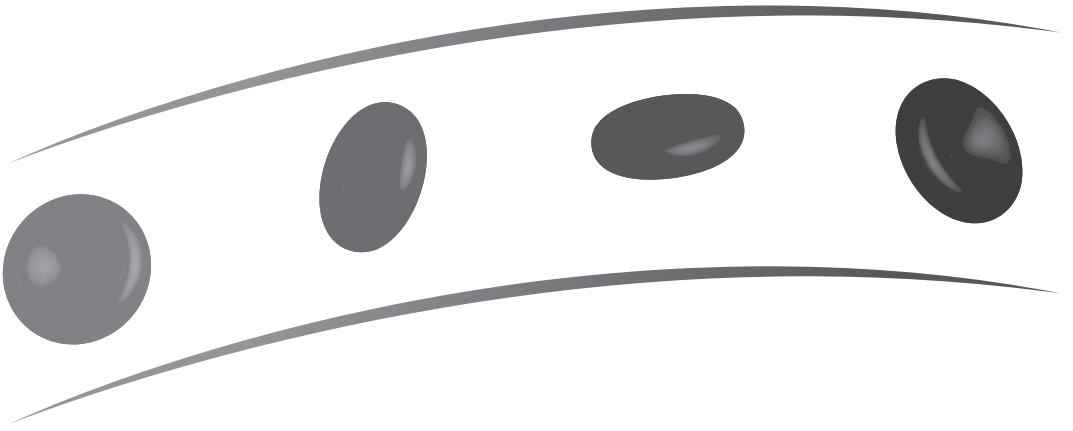

CHAPTER 10

General conclusions and discussion

Impaired perfusion of the microcirculation is an important contributor to acute organ dysfunction. However, imaging of microcirculatory perfusion in patients was not possible until the last decade. In the current thesis, we studied microvascular perfusion during cardiac surgery with cardiopulmonary bypass. In particular, we focused on the mechanisms underlying impaired perfusion of the microcirculation, and we evaluated the use of pulsatile flow during cardiopulmonary bypass and reduction of vascular leakage for improving microcirculatory perfusion in on-pump cardiac surgery.

Using non-invasive imaging techniques of the microvasculature in patients undergoing coronary artery bypass grafting, we investigated the alterations in microcirculatory perfusion, including glycocalyx integrity and heterogeneity of blood flow, in a clinical setting. We compared microvascular perfusion in patients subjected to on-pump and off-pump cardiac surgery in order to detect the effects of cardiopulmonary bypass on the microcirculation. We additionally aimed to improve microcirculatory perfusion during on-pump cardiac surgery, randomizing patients into undergoing cardiopulmonary bypass with pulsatile flow or the conventional nonpulsatile flow.

Subsequently, we evaluated the effect of plasma from patients undergoing cardiac surgery with cardiopulmonary bypass on the barrier function of the vascular wall, in order to investigate the development of vascular leakage during cardiac surgery. Cultured endothelial cells were exposed to plasma from patients undergoing cardiac surgery with pulsatile or nonpulsatile flow during cardiopulmonary bypass and the effects on the endothelial barrier function were studied.

A rat cardiopulmonary bypass model with a cremaster muscle preparation for intravital microscopy of the microvasculature was developed and the role of hemodilution in the development of microcirculatory perfusion disturbances during cardiopulmonary bypass was investigated. Finally, rats undergoing cardiopulmonary bypass were treated with imatinib in order to reduce vascular leakage, and we studied the effects on the perfusion of the microcirculation and on markers of organ injury markers.

Mechanisms underlying microcirculatory disturbances during cardiac surgery with cardiopulmonary bypass

Onset of cardiopulmonary bypass during cardiac surgery leads to impairment of microcirculatory perfusion. The occurrence of microcirculatory disturbances is thought to result in acute organ dysfunction [1-3] and the absence of early microcirculatory recovery is a good predictor of ICU mortality in critically ill patients [4,5]. However, monitoring techniques for microcirculatory perfusion in patients have only recently become available. The current knowledge regarding the mechanisms underlying disturbed microvascular perfusion during cardiac surgery is therefore limited.

In **chapter 3**, we have shown that coronary artery bypass surgery with cardiopulmonary bypass leads to impaired perfusion of the microcirculation during and early after surgery, whereas similar surgical procedures without use of cardiopulmonary bypass are associated with preserved perioperative microvascular perfusion. Whether this difference can be attributed to the inflammatory response resulting from exposure to the extracorporeal circuit, or hemodilution in the group undergoing cardiopulmonary bypass was unclear. To investigate the effect of hemodilution in depth, we showed in **chapter 8** in an animal model for cardiopulmonary bypass that hemodilution alone only leads to a minor and transient decrease in the number of perfused vessels in the microcirculation. The impact of hemodilution on the microcirculation was significantly lower when compared to animals exposed to cardiopulmonary bypass with similar hematocrit levels as in the hemodiluted animals. From our experiments, we conclude that perfusion disturbances in the microcirculation during cardiopulmonary bypass occur mainly due to the inflammatory activation by the extracorporeal circuit and consequent endothelial barrier dysfunction and vascular leakage. However, hemodilution may further aggravate tissue oxygenation during microcirculatory perfusion alterations in on-pump cardiac surgery. We therefore confirm the hypothesis as presented in the Introduction section. This two-hit model for microcirculatory impairment during cardiac surgery with cardiopulmonary bypass is presented in Figure 1.

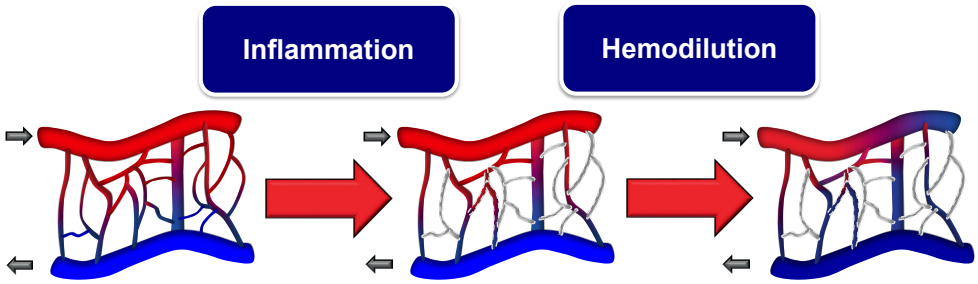

Figure 1 Two-hit model of impaired microcirculatory perfusion during cardiac surgery with cardiopulmonary bypass. The inflammatory response induced by blood contact activation by the extracorporeal circuit results in reduced capillary perfusion. Hemodilution additionally interferes with oxygen delivery and poses an additional threat to tissue oxygenation. Direction of blood flow is indicated by grey arrows. Oxygenated and deoxygenated blood are presented in red and blue, respectively, whereas non-perfused vessels are shown in grey.

This concept is supported by the observation that hemodilution is a risk factor for acute kidney injury following cardiac surgery with cardiopulmonary bypass [6]. Hemodilution not only leads to a reduction in oxygen delivery, but may additionally reduce the number of perfused capillaries through decreased blood viscosity, and interferes with the role of hemoglobin as a sensor of blood flow distribution in the microcirculation [7,8].

The important role of the endothelium for microvascular perfusion was demonstrated in **chapter 4**. Nonpulsatile flow leads to reduced shear stress on the luminal side of the endothelium as compared to pulsatile flow, which results in reduced endothelial nitric oxide synthase activity and increased endothelin-1 production. Both are associated with increased prothrombogenicity and arteriolar vasoconstriction resulting in reduced microvascular perfusion [9,10]. In **chapter 4**, we showed that minimizing the time that the body is exposed to nonpulsatile flow during cardiopulmonary bypass leads to improved postoperative microcirculatory perfusion when compared to nonpulsatile flow throughout aortic cross-clamp time. Patients undergoing pulsatile and nonpulsatile flow both show an initial disturbance in microvascular perfusion caused by the inflammatory response to the extracorporeal circuit and hemodilution. There was however a profound postoperative recovery of microcirculatory perfusion following pulsatile flow, which was absent in the nonpulsatile flow group. This may be attributed to reduced disturbance of endothelial cell function with pulsatile flow as compared to nonpulsatile flow. In **chapter 6** it became obvious in our endothelial barrier function bioassay that the beneficial effects of pulsatile flow on the endothelium seem not to be mediated through circulating factors, and appear to be a direct effect of shear stress on the vascular wall. This is confirmed in a study of Pinaud et al., where it was shown that the exposure of resistance arteries to nonpulsatile flow induces an inflammatory reaction with generation of reactive oxygen species and proinflammatory cytokines in the vascular wall, even in the absence of blood components [11].

Indeed, in **chapter 8**, we demonstrated that microcirculatory impairment was paralleled by increased expression of endothelial activation molecules in the renal and pulmonary vasculature. From these data it was however not clear whether endothelial activation is a contributor to or a consequence of reduced microcirculatory perfusion due to the lack of causality. In **chapter 9** we therefore investigated the role of endothelial barrier protection in microcirculatory perfusion disturbances during cardiopulmonary bypass (vascular leakage will be discussed in more detail in a following section). In this chapter we demonstrated that imatinib treatment inhibited vascular leakage, which is a downstream effect of endothelial activation, and was able to preserve microvascular perfusion during cardiopulmonary bypass. Endothelial adhesion molecule expression was largely similar between imatinib treated and untreated groups, suggesting that the inflammatory response elicited by cardiopulmonary bypass led to similar endothelial activation in both groups. The observation that inhibition of a downstream effect of endothelial activation can preserve microcirculatory perfusion confirms the important role of endothelial activation in the development of microvascular perfusion disorders in cardiac surgery with cardiopulmonary bypass.

Disruption of the endothelial glycocalyx may additionally contribute to endothelial activation. Although the endothelial glycocalyx has been difficult to investigate in vivo, measurements of plasma concentrations of glycocalyx components suggest that the glycocalyx is injured

during cardiac surgery with cardiopulmonary bypass [12,13]. Different stimuli may lead to shedding of glycocalyx components, including inflammation, trauma, reperfusion injury, hyperglycemia, or hypervolemia [14]. All of these factors may exist during cardiac surgery with cardiopulmonary bypass, thereby contributing to injury of the endothelial glycocalyx. The causal consequence of endothelial glycocalyx disruption for microvascular perfusion has been investigated in an experimental study by Cabrales et al. [15]. They have shown that infusion of hyaluronidase, which cleaves the glycocalyx component hyaluronan, leads to a reduction in the number of perfused capillaries in hamsters [15]. No clinical studies thus far had evaluated the effect of glycocalyx alterations on microcirculatory perfusion. In **chapter 7** we used a noninvasive measurement of glycocalyx thickness in sublingual capillaries. Here we showed that glycocalyx thickness is correlated to perfused capillary density, suggesting that endothelial glycocalyx disruption leads to impaired microvascular perfusion in patients undergoing cardiac surgery with cardiopulmonary bypass. Although this observation does not provide concluding evidence with regard to the role of the endothelial glycocalyx in the development of acute microvascular perfusion disorders, our study supports a role for the endothelial glycocalyx in this pathophysiological process, and supports the search for protective strategies of the endothelial surface layer in patients undergoing cardiac surgery with cardiopulmonary bypass.

In **chapter 5**, we described the presence of a microcirculatory shunting phenomenon after onset of nonpulsatile cardiopulmonary bypass. Microvascular shunting was characterized by capillary red blood cell velocities during cardiopulmonary bypass that were five to ten times increased as compared to baseline. It has been calculated that the capillary blood velocities as observed during cardiopulmonary bypass are associated with impaired oxygen offloading [16]. This was confirmed by our measurements, showing that high capillary blood velocities were paralleled by a low oxygen extraction rate and a high mixed venous oxygen saturation. Although microvascular shunting per se does not lead to tissue hypoxia, in combination with the observed increase in heterogeneity of microvascular perfusion, blood is directed away from the remaining capillaries. Indeed, systemic oxygen consumption during cardiopulmonary bypass was decreased by 35% as compared to baseline, suggesting that a microcirculatory mismatch of oxygen supply and demand resulted in impaired tissue oxygenation. Although we did not investigate the cause of microvascular shunting, we did not observe similar alterations in microvascular flow profile of patients undergoing off-pump surgery. The origin of this shunting phenomenon should be sought in the inflammatory response or hemodilution associated with extracorporeal circulation, since systemic hemodynamics were similar between patients undergoing cardiac surgery with or without cardiopulmonary bypass. It remains to be investigated whether interventions aimed at preservation of microcirculatory perfusion during cardiopulmonary bypass additionally reduce shunting in the microcirculation and consequently improve efficiency in tissue oxygenation.

The role of vascular leakage in microcirculatory disturbances in cardiac surgery with cardiopulmonary bypass

In **chapter 9**, we evaluated the use of imatinib in rats undergoing cardiopulmonary bypass. Imatinib is a tyrosine kinase inhibitor of multiple intracellular pathways, including c-Abl, Abl-related gene (Arg), platelet derived growth factor receptor (PDGFR), c-Kit and discoid domain receptor-1 [17]. It has been registered for the treatment of chronic myeloid leukemia or gastro-intestinal stromal tumors with a positive Bcr-Abl mutation. Recently, it has been shown that imatinib inhibits endothelial barrier dysfunction in septic mice and consequently reduces vascular leakage [18]. In this chapter, we aimed to preserve microvascular perfusion in rats undergoing cardiopulmonary bypass using imatinib for maintenance of endothelial barrier integrity. Indeed, vascular leakage was reduced in multiple organs and fluid resuscitation requirements were lower following imatinib treatment as compared to untreated animals. Additionally, the number of perfused capillaries remained unaltered after onset of cardiopulmonary bypass following imatinib treatment, demonstrating the important role of vascular leakage as a cause of acute microcirculatory perfusion disturbances. Although the specific pathway by which imatinib reduces vascular leakage was not investigated in our study, previous studies in mice models of sepsis and acute lung injury have identified Arg and c-Abl as important pathways for endothelial barrier dysfunction [18,19]. In addition, perioperative fluid accumulation is a risk factor for long intensive care unit stay and postoperative complications following cardiac surgery, but the exact link between these two remained unrevealed [20]. According to our findings, impaired microvascular perfusion may be the central factor leading to postoperative organ dysfunction as a consequence of vascular leakage and concomitant interstitial edema.

Perioperative fluid overload is usually the consequence of vascular leakage, necessitating continued fluid resuscitation in the early postoperative period. In **chapter 9**, we also demonstrated a 75% reduction in fluid resuscitation requirements following imatinib treatment when compared to control animals, while hematocrit levels remained similar between groups. These observations demonstrate the powerful effect of imatinib in inhibiting vascular leakage. Currently, no therapies exist that specifically reduce vascular leakage in cardiac surgery. Although glucocorticoids may reduce extravascular fluid content following cardiac surgery, they have been associated with adverse effects including susceptibility to infections, hyperglycemia or gastrointestinal bleeding [21,22]. A beneficial effect of glucocorticoids on clinical outcome has however not been demonstrated in two large randomized controlled trials [22,23]. A more selective inhibitor of endothelial barrier dysfunction, such as imatinib, may therefore be used to reduce postoperative complications associated with fluid overload and contribute to a reduction in the development of perioperative complications following cardiac surgery.

Monitoring microcirculation perfusion, a tool for the clinician?

Although the relevance of microcirculatory perfusion in critical ill patients and patients undergoing major surgery has been well demonstrated over the last 15 years, use of microcirculatory monitoring techniques have remained confined to a research setting instead of development towards clinical monitoring devices. Recently, several investigators have promoted the use of microcirculatory guided fluid therapy [24-26]. The impairment of microcirculatory perfusion during inflammatory disorders including cardiac surgery with cardiopulmonary bypass or septic shock has appeared to be a complex problem and often occurs in the absence of deranged systemic hemodynamics. Factors contributing to acute microcirculatory perfusion disturbances in these patient populations include endothelial cell dysfunction, endothelial glycocalyx disruption, leukocyte, platelet and complement activation, hemodilution and reduced red blood cell deformability. Moreover, most studies included subjects with macrocirculatory variables such as mean arterial pressure and cardiac index within the normal range. It would therefore be highly surprising if simple interventions to optimize macrocirculatory hemodynamics, such as fluid therapy or the administration of additional vasoactive medication, would improve a compromised microcirculatory perfusion and, more importantly, clinical outcome. Indeed, van der Voort et al. failed to show a beneficial effect of a goal directed therapy based on observations of the microcirculation using macrocirculatory interventions in patients with severe sepsis or septic shock [27]. Similarly, Boerma et al. showed that the vasodilator nitroglycerin did not improve microvascular perfusion in severe septic patients but instead was associated with a trend towards an increase in mortality [28]. Although multiple techniques for microcirculatory perfusion in patients are now available for over a decade, none of those have acquired a place at bedside as a tool for the clinician. This lack of success for microcirculatory imaging as a clinical tool has multiple explanations.

First, microcirculatory videomicroscopy is an analogue technique providing images instead of numbers, which makes its use as a continuous monitoring tool unfeasible. Moreover, cardiac surgery with cardiopulmonary bypass and sepsis are associated with heterogeneity of microvascular perfusion, and this requires evaluation of multiple measurement sites [4,29]. Additionally, the level of heterogeneity itself may be as important for the assessment of microvascular perfusion as the number of perfused vessels that is usually measured [30].

Furthermore, in most patients microcirculatory perfusion measurements are restricted to the sublingual, conjuctival or skin microvasculature. Although systemic inflammation affects all vascular beds, differences in microvascular regulation and endothelial cells architecture and function between organs exist [31]. The relation between sublingual and gastro-intestinal mucosal microcirculatory derived parameters appears good [32,33], but caution must be exerted to interpret localized microcirculatory disorders for systemic microvascular perfusion. Another limitation of clinical microcirculatory perfusion imaging is that results may be very

operator-dependent, unless sufficient experience has been obtained. Finally, and most importantly, currently no rational therapies exist for specific optimization of microvascular perfusion. Consequently, if poor microcirculatory perfusion is observed, macrocirculatory interventions including fluid therapy, vasopressors, inotropes or vasodilators are suggested to improve microcirculatory perfusion [27]. However, it seems more rational that the use of these interventions are guided by macrohemodynamic parameters as preload, systemic vascular resistance, myocardial contractility or heart rate. Within a normal range of systemic hemodynamics, fluid therapy and vasoactive medication hardly influence microcirculatory perfusion parameters in patients in septic shock [34]. The absence of therapeutics acting specifically on the microcirculation therefore limits the use of microvascular monitoring in the clinic.

Until these limitations have been addressed, microcirculatory perfusion monitoring will not routinely be used as a clinical monitoring device. However, due to the increased use of microvascular imaging as a research tool we are able to unravel the pathophysiology of microcirculatory dysfunction and the evaluation of new therapeutic interventions for optimization of microcirculatory perfusion in acute disease.

Microcirculatory perfusion disturbances in cardiac surgery: affecting outcome?

Adequate perfusion of the microcirculation is necessary for sufficient tissue oxygenation. However, since current measurement techniques for microvascular perfusion are mostly limited to a research setting, no reference values or cut-off values associated with poor outcome exist. In patients undergoing major abdominal surgery, septic patients and patients with cardiogenic shock, impaired microcirculatory perfusion was associated with worse clinical outcome [4,5,35,36]. Moreover, the early response of the microcirculation to therapy appears to be predictive of survival in septic patients admitted to the emergency department [37]. Although it has been shown that goal-directed hemodynamic optimization leads to improved microcirculatory perfusion and reduced acute kidney injury in patients undergoing major abdominal surgery [38], no relation between perfusion of the microcirculation and clinical outcome has currently been demonstrated in patients undergoing cardiac surgery with cardiopulmonary bypass.

Most studies investigating microcirculatory perfusion in cardiac surgery have studied patients undergoing relatively low-risk cardiac surgical procedures with low complication rates, which hinders detection of an association of microcirculatory perfusion disturbances with acute organ dysfunction or length of hospital stay. However, from the abovementioned studies it became clear that impaired microcirculatory perfusion is associated with increased morbidity and mortality, and microvascular disturbances during cardiac surgery therefore deserves attention. In our rat cardiopulmonary bypass model as described in **chapter 8**, we indeed show that cardiopulmonary bypass leads to increased microcirculatory perfusion

disturbances that are paralleled by increased renal immunohistological injury markers. In **chapter 9**, rats undergoing cardiopulmonary bypass following treatment with imatinib showed preservation of microcirculatory perfusion throughout the experimental protocol. This was paralleled by reduced markers for renal, pulmonary and intestinal injury when compared to untreated animals. These data suggest that impaired microvascular perfusion contributes to organ dysfunction following cardiopulmonary bypass, and that preservation of microcirculatory perfusion may improve clinical outcome. However, whether this holds true for patients should be investigated in larger clinical trials.

Methodological considerations

In **chapter 3, 4, 5 and 7**, we used sublingual sidestream darkfield imaging in order to assess microcirculatory perfusion. The technique is based on the absorbance of green light by erythrocytes and reflection of the light by the remainder of the tissue, visualizing red blood cells in superficial arterioles, capillaries and venules. Several vascular beds are suitable for assessment with SDF imaging, with the sublingual vasculature being most used in clinical studies, due to its accessibility and relatively central location. It has been shown that alterations in perfusion of the sublingual microvasculature correlate well with alterations in perfusion parameters of the gastric of intestinal mucosa [32,33].

Although microvascular beds show different anatomical characteristics or regulatory mechanisms between or within organs [39], the inflammatory activation of blood by the extracorporeal circuit provides a systemic hit to the microvasculature, and we assume that all vascular beds will be affected. Indeed, impairment of microcirculatory perfusion in the cremaster muscle of the rat during a model of cardiopulmonary bypass shows a similar course to sublingual microcirculatory alterations observed in patients undergoing nonpulsatile cardiopulmonary bypass. Alternative techniques, such as reflectance spectrophotometry, near-infrared spectroscopy or capnometry provide information about microcirculatory perfusion using derived parameters as regional oxygenation or partial pressure of carbon dioxide. These techniques all provide a numerical outcome as the resultant of all combined microcirculatory perfusion alterations within a region of interest. However, this numerical outcome disregards any heterogeneity within that region, although we showed in **chapter 5** that this is an important characteristic of microcirculatory perfusion disturbances. Although in vivo assessment of organ capillary perfusion with minimal alterations induced by the measurement technique would be preferred, to our knowledge no such technique currently exists.

The electrical cell-substrate impedance sensing (ECIS) system used in **chapter 6** is a technique to measure impedance of cultured cells in vitro. In this chapter we used human umbilical vein endothelial cells (HUVECs) cultured in wells in order to measure electrical resistance across the confluent monolayers as a measure of endothelial barrier function. In contrast to in vivo endothelial cells, the cultured HUVECs do not have an endothelial

glycocalyx, which significantly contributes to the endothelial barrier function in vivo [40]. Moreover, the absence of pulsatile flow-induced shear stress may induce an inflammatory activation of the endothelium, even in the absence of blood components [11]. However, the ECIS technique has often been used for evaluation of endothelial barrier function and its response to interventions [18,41]. With careful interpretation of the results, it provides a highly interesting technique for the evaluation of novel treatments on the preservation of endothelial barrier function.

In **chapter 8 and 9**, we used a cardiopulmonary bypass model in the rat in order to investigate the effect of interventions on microcirculatory function. The generalization of these experimental findings for the human setting remains a subject of debate. Several characteristics of cardiac surgical procedures with cardiopulmonary bypass are lacking in our model, including heparin coating of the extracorporeal system, surgical trauma, central cannulation or aortic cross clamping. However, our model includes the main pathophysiological factors that may influence microcirculatory perfusion, including hemodilution, contact activation of blood, loss of pulsatile blood flow and mild hypothermia. Moreover, cardiopulmonary bypass was possible despite the absence of cardioplegic arrest in our model, and this allowed discontinuation of mechanical ventilation during cardiopulmonary bypass. No signs of hypoxic coronary perfusion were present in the post-cardiopulmonary bypass period. Although cardiopulmonary bypass as conducted in our model may not completely reflect a state-of-the-art on-pump cardiac surgical procedure, the main pathophysiological principles of cardiopulmonary bypass are all present in our model. Moreover, microcirculatory perfusion parameters in our rat model showed a similar perioperative course as previously found in patients undergoing cardiac surgery with cardiopulmonary bypass. The current rat model therefore provides a good opportunity to investigate novel therapies for preservation of microvascular perfusion and the reduction of organ injury following cardiopulmonary bypass.

Future directions

In **chapter 4**, we showed that the use of pulsatile flow during cardiopulmonary bypass leads to improved microcirculatory perfusion in the early postoperative period as compared to nonpulsatile flow. Whether preservation of the microvascular perfusion translates into improvement of clinical outcome remains to be determined. Two large randomized controlled trials have shown reduced morbidity and mortality with pulsatile versus nonpulsatile flow during cardiopulmonary bypass, although these two older trials that may not completely represent contemporary clinical practice [42,43]. The benefit of pulsatile flow during cardiopulmonary bypass may be most outspoken in patients with extensive comorbidities undergoing high-risk cardiac surgery, since this population is at risk for developing postoperative organ dysfunction associated with impaired microvascular perfusion. A novel randomized controlled trial will investigate whether the use of pulsatile flow during cardiopulmonary bypass is indeed a low-risk intervention that contributes to decreased postoperative acute organ dysfunction.

The role of the glycocalyx in the origin of dysfunction of the endothelial barrier in patients undergoing cardiac surgery with cardiopulmonary bypass should be further investigated. An intact endothelial glycocalyx is an important contributor to the barrier of the vascular wall [44]. In **chapter 7**, we have shown the relation between the dimensions of the endothelial glycocalyx and the density of perfused vessels in the microcirculation, suggesting that injury to the endothelial glycocalyx may result in impaired microvascular perfusion during cardiac surgery with cardiopulmonary bypass. This is possibly mediated by increased vascular leakage following glycocalyx degradation. It will be interesting to study interventions protecting or facilitating repair of the endothelial glycocalyx in our rat cardiopulmonary bypass model, as antithrombin III, albumin, hydrocortisone or sulodexide, which contains components of the endothelial glycocalyx [14,44].

Finally, we demonstrated the important role of endothelial barrier dysfunction in the development of microcirculatory perfusion disorders in **chapter 9**. Treatment with imatinib was used to reduce endothelial barrier dysfunction, which was paralleled by improved perfusion and oxygenation in the microcirculation and reduced markers of organ injury following cardiopulmonary bypass in a rat model. Imatinib is a drug registered for the treatment of chronic myeloid leukemia, and extensive experience has been generated with long-term treatment in the last decades. Future studies should focus on the effects and side effects of perioperative use of imatinib in human patients in order to improve microcirculatory perfusion and reduce organ dysfunction following cardiac surgery with cardiopulmonary bypass.

Conclusions

The current thesis has demonstrated that microcirculatory perfusion is impaired during and after cardiac surgery, and this can be attributed mainly to inflammatory endothelial barrier dysfunction and consequent vascular leakage. Concomitant hemodilution may additionally contribute to reduced microvascular perfusion and oxygenation in on-pump cardiac surgery. We showed that the use of pulsatile flow during cardiopulmonary bypass improves postoperative microvascular perfusion as compared to nonpulsatile flow. Imatinib treatment reduced endothelial barrier dysfunction and vascular leakage in our rat model for cardiopulmonary bypass and resulted in preservation of microcirculatory perfusion and oxygenation during and after extracorporeal circulation. Moreover, imatinib treatment resulted in reduced markers of renal, pulmonary and intestinal injury after cardiopulmonary bypass. Based on our findings, reduction of vascular leakage and use of pulsatile flow during cardiopulmonary bypass are promising interventions for the prevention of postoperative complications in patients at risk for organ failure following cardiac surgery with cardiopulmonary bypass. We suggest that the effect of pulsatile flow during cardiopulmonary bypass on postoperative organ dysfunction should be evaluated in a randomized controlled trial and that the safety and feasibility of

imatinib treatment for patients undergoing cardiac surgery with cardiopulmonary bypass should be assessed in a pilot study.

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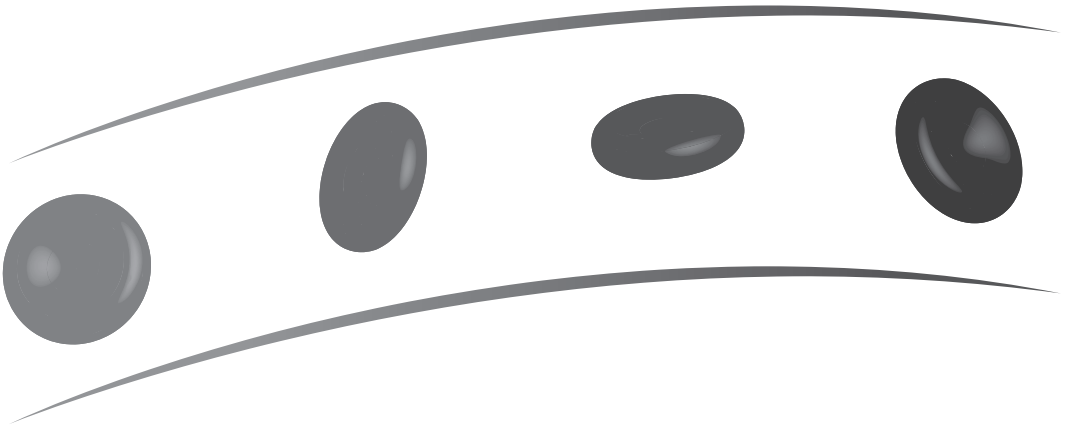

English summary

In this thesis we aimed to investigate the mechanisms underlying impaired microcirculatory perfusion during cardiac surgery with use of cardiopulmonary bypass. Additionally, we evaluated two treatment strategies in order to protect the microcirculation from harmful stimuli induced by cardiopulmonary bypass: a pulsatile flow modality during cardiopulmonary bypass and the reduction of vascular leakage through a reduction of endothelial permeability.

Chapter 1 provides an introduction on cardiac surgery and describes postoperative morbidity related to the use of cardiopulmonary bypass. Moreover, the microcirculation and its relevance for the perioperative physician are described.

Chapter 2 provides a review of the alterations that occur in the microcirculation during cardiac surgery with or without the use of cardiopulmonary bypass. Microcirculatory monitoring techniques available for the clinician are described. Furthermore, factors that may contribute to disturbances of microcirculatory perfusion during cardiac surgery, including hemodilution, mild hypothermia, use of cardiopulmonary bypass and nonpulsatile flow are discussed.

In **chapter 3**, we have assessed the perfusion characteristics of the sublingual microcirculation during off-pump cardiac surgery and on-pump cardiac surgery using Sidestream Darkfield (SDF) imaging. Microvascular perfusion was well maintained during and after off-pump surgery, whereas patients undergoing on-pump surgery showed a 20% decrease in the density of perfused capillaries after onset of cardiopulmonary bypass. No recovery of microcirculatory perfusion was observed at intensive care unit admission in the on-pump group, despite normalization of temperature and hematocrit. Interestingly, we found no correlation between microcirculatory and macrocirculatory parameters. We concluded that off-pump surgery was associated with preserved microcirculatory perfusion, whereas on-pump surgery led to a reduction in capillary perfusion which did not recover in the early postoperative phase.

In **chapter 4**, we compared the use of pulsatile flow with conventional, nonpulsatile flow during cardiopulmonary bypass in patients undergoing on-pump cardiac surgery in a single-blinded randomized trial. It has been demonstrated that pulsatile flow may have a protective effect on the endothelium as compared to nonpulsatile flow and we therefore hypothesized that pulsatile flow during cardiopulmonary bypass preserves capillary perfusion during cardiac surgery. The effects on capillary perfusion were studied using SDF imaging of the sublingual mucosa at multiple time points during surgery and at intensive care unit admission. Pulsatile flow did not prevent the initial decrease of capillary perfusion during cardiopulmonary bypass.

However, recovery of microvascular perfusion after disconnection from cardiopulmonary bypass was improved in the pulsatile flow group when compared to patients exposed to nonpulsatile flow. No difference in plasma inflammatory parameters was observed. Systemic oxygen consumption and oxygen extraction ratio improved during cardiopulmonary bypass only in the group undergoing pulsatile flow, which may indicate a reduction in microvascular shunting. The positive effect of pulsatile flow on capillary perfusion after cardiopulmonary bypass most likely depends on an endothelial cell-mediated effect instead of a direct effect of the pulse, given the delayed recovery of the microcirculation postoperatively.

In **chapter 5**, the blood flow characteristics in the sublingual microcirculation during on-pump and off-pump cardiac surgery were assessed. We investigated the hypothesis that acute physiological alterations during extracorporeal circulation lead to systemic shunting in the microcirculation. Our data showed that cardiopulmonary bypass is associated with increased microvascular perfusion heterogeneity, and this phenomenon persists postoperatively. Moreover, one-third of the capillaries revealed extremely high red blood cell velocities, incompatible with normal oxygen offloading. In parallel, the arteriovenous oxygen content difference and systemic oxygen consumption decreased, indicating the presence of shunting of blood in the systemic microcirculation during and after cardiopulmonary bypass. We observed no increased microcirculatory heterogeneity or microvascular shunting during off-pump surgery. From these findings we conclude that systemic microvascular shunting is present in patients undergoing cardiac surgery with cardiopulmonary bypass and this is associated with reduced systemic oxygen extraction, while this phenomenon is not present during off-pump surgery.

In **chapter 6**, we investigated whether cardiopulmonary bypass is associated with loss of endothelial barrier function in an in vitro endothelial cell culture that was exposed to plasma from patients undergoing pulsatile or nonpulsatile cardiopulmonary bypass. Transendothelial resistance was assessed using electric cell-substrate impedance sensing in order to quantify permeability of the endothelial cell monolayer. Plasma sampled after cardiopulmonary bypass led to an increase in endothelial permeability as compared to plasma from baseline. This was paralleled by an increase in endothelial cell activation markers after cardiopulmonary bypass. Hemodilution did not have an effect on endothelial permeability, nor did the use of pulsatile flow as compared to nonpulsatile flow. The current investigation demonstrates that components in patient plasma following on-pump cardiac surgery induce an increased loss of endothelial barrier function in an in vitro model. This effect may be related to the increase in inflammatory and endothelial activation markers.

Chapter 7 describes the relationship between the endothelial glycocalyx and microcirculatory perfusion during cardiac surgery. In patients undergoing off-pump cardiac surgery or on-pump cardiac surgery with pulsatile or nonpulsatile flow, we have investigated endothelial glycocalyx dimensions using a sublingual imaging technique assessing glycocalyx dimensions. In both groups undergoing on-pump cardiac surgery, glycocalyx dimensions decreased after onset of cardiopulmonary bypass, whereas a recovery after disconnection from cardiopulmonary bypass was only observed following pulsatile CPB. Off-pump surgery was not associated with reduced glycocalyx dimensions. Moreover, we found a correlation between glycocalyx dimensions and perfused vessel density in the microcirculation, suggesting that reduced endothelial glycocalyx dimensions are associated with impaired capillary perfusion in patients undergoing cardiac surgery.

In **chapter 8**, a rat model of cardiopulmonary bypass with a preparation of the cremaster muscle for investigation of microcirculatory perfusion is described. The role of hemodilution in the development of microcirculatory alteration during cardiopulmonary bypass was investigated. Rats underwent either cardiopulmonary bypass, acute hemodilution to a similar hematocrit or a sham procedure. Microcirculatory perfusion remained unaltered in rats undergoing a sham procedure. Onset of cardiopulmonary bypass led to a 40% reduction in perfused capillaries, which showed only a small recovery one hour after disconnection from cardiopulmonary bypass. Acute hemodilution alone led to a minor and temporary decrease in microvascular perfusion. Cardiopulmonary bypass induced increased inflammatory and endothelial activation as compared to hemodilution. Additionally, renal injury as assessed histologically was increased following cardiopulmonary bypass. We concluded that hemodilution cannot fully explain impaired microcirculatory perfusion induced by cardiopulmonary bypass, and may be only a minor and temporary contributor.

In **chapter 9**, we hypothesized that vascular leakage during cardiopulmonary bypass is a major contributor to disturbed capillary perfusion, and that reduction of vascular leakage preserves microvascular perfusion. Before cardiopulmonary bypass, rats were randomized into treatment with imatinib, known for its protecting effects on the endothelial barrier in septic conditions, or placebo. Cremaster muscle microcirculatory perfusion showed a significant decrease following onset of cardiopulmonary bypass following placebo treatment, whereas imatinib led to preservation of microvascular perfusion. In parallel, vascular leakage decreased in multiple organs under imatinib treatment, and was paralleled by reduced fluid requirements during and after cardiopulmonary bypass. Moreover, markers of renal and pulmonary injury were lower in rats treated by imatinib as compared to placebo. The current results suggest that vascular leakage serves as an important contributor to impaired microvascular perfusion during cardiopulmonary bypass, and that reduction of endothelial

barrier dysfunction improves microcirculatory perfusion and organ injury markers and reduces fluid resuscitation requirements.

In **chapter 10** the main results of the current thesis are discussed, as well as the methodological considerations for the studies performed and possible future directions for further research. We proposed a two-hit model for the development of acute microcirculatory perfusion disturbances and impaired tissue oxygenation during cardiac surgery with cardiopulmonary bypass. The inflammatory response generated by the exposure of blood to the extracorporeal circuit leads to endothelial activation and vascular leakage resulting in a reduced number of perfused capillaries. Concomitant hemodilution then further impairs tissue oxygenation through reduced oxygen delivery. We concluded that microcirculatory dysfunction during cardiac surgery with cardiopulmonary bypass is mainly to be attributed to increased vascular leakage due to inflammatory endothelial barrier dysfunction. Both pulsatile flow during cardiopulmonary bypass and imatinib treatment are promising interventions for improvement of microcirculatory perfusion and reduction of acute postoperative organ dysfunction in cardiac surgery with cardiopulmonary bypass.

Nederlandse samenvatting

Het doel van het onderzoek zoals beschreven in dit proefschrift was om de mechanismen te bestuderen die ten grondslag liggen aan verstoringen in de doorbloeding van de haarvaten (microcirculatie) tijdens hartoperaties waarbij gebruik wordt gemaakt van een hartlongmachine.
De hartlongmachine neemt tijdens een hartoperatie tijdelijk de functie van de longen en het hart over. De hartlongmachine pompt zuurstofrijk bloed naar het lichaam en voert zuurstofarm bloed af. Het zuurstofarme bloed wordt in de hartlongmachine weer voorzien van zuurstof. Een nadelig effect van de hartlongmachine is dat het bloed van patiënten wordt verdund, patiënten afkoelen en de pulsatiele bloedstroom die het hart genereert afwezig is. In sommige gevallen kan de hartoperatie plaatsvinden zonder gebruik te maken van een hartlongmachine, de zogenaamde off-pump procedures.
In het proefschrift worden twee interventies bestudeerd die bescherming kunnen bieden tegen de schadelijke effecten van het gebruik van de hartlongmachine op de microcirculatie. De eerste interventie betrof het aanbieden van pulsatiele flow tijdens het gebruik van de hartlongmachine in patiënten die een hartoperatie ondergingen. De tweede interventie bestond uit het reduceren van vaatlekkage door het beschermen van de endotheliale barrière functie in een dierexperimenteel model.

In **Hoofdstuk 1** wordt een beschrijving gegeven van verschillende aspecten van hartchirurgie. Daarnaast wordt ingegaan op postoperatieve complicaties die kunnen optreden ten gevolge van het gebruik van de hartlongmachine. Tot slot wordt de microcirculatie en de relevantie van adequate microcirculatoire perfusie voor de perioperatieve arts beschreven.

Hoofdstuk 2 geeft een overzicht over de veranderingen die plaatsvinden in de microcirculatie tijdens hartchirurgie met of zonder gebruik van de hartlongmachine. Daarnaast worden de technieken beschreven die beschikbaar zijn voor het bestuderen van de microcirculatie in de klinische setting. Als laatste komen verschillende perioperatieve factoren aan bod die de microcirculatoire perfusie kunnen verstoren tijdens hartchirurgie, zoals verdunning (hemodilutie), milde onderkoeling (hypothermie), en de toepassing van een niet-pulsatiele bloedstroom tijdens het gebruik van de hartlongmachine.

In **Hoofdstuk 3** hebben we de doorstroming van de microcirculatie aan de onderkant van de tong meten bij patiënten die een hartoperatie ondergingen en wel of niet waren aangesloten aan een hartlongmachine. De doorbloeding van de microcirculatie werd door middel van een kleine camera gemeten. Wanneer patiënten niet werden aangesloten aan de hartlongmachine, de zogenaamde off-pump procedures, bleef de doorbloeding van de

microcirculatie relatief constant. Wanneer patiënten wel aan de hartlongmachine werden aangesloten ter ondersteuning van hart- en longfunctie verminderde de doorbloeding van de microcirculatie met 20%. Daarnaast bleek dat de verminderde doorbloeding van de microcirculatie in deze patiënten niet herstelde na de operatie, ondanks dat de patiënten weer afgekoppeld waren van de hartlongmachine. De verminderde doorbloeding van de microcirculatie bleek niet gerelateerd zijn aan veranderingen in bloeddruk of de pompfunctie van het hart tijdens de operatie. Uit deze studie concludeerden we dat hartoperaties waarbij gebruik wordt gemaakt van de hartlongmachine gepaard gaan met verstoringen van de doorbloeding van de microcirculatie, terwijl deze verstoringen niet optreden als patiënten off-pump chirurgie ondergaan.

In **Hoofdstuk 4** vergeleken we de effecten twee verschillende methoden waarop de hartlongmachine tijdens een hartoperatie bloedstroom genereert op de doorbloeding van de microcirculatie. De eerste methode betrof een pulsatiele bloedstroom, de tweede methode betrof de gebruikelijke niet-pulsatiele bloedstroom. Uit de literatuur was eerder gebleken dat het gebruik van de pulsatiele bloedstroom een beschermend effect op de bloedvaten zou kunnen hebben. In dit onderzoek vroegen wij ons daarom af of het gebruik van een pulsatiele bloedstroom ook een voordelig effect op de doorbloeding van de microcirculatie zou kunnen hebben in patiënten die een hartoperatie ondergaan. De doorbloeding van de microcirculatie werd door middel van een kleine camera gemeten. Tijdens het onderzoek bleek dat een pulsatiele bloedstroom tijdens het gebruik van de hartlongmachine niet kon voorkomen dat de doorbloeding van de microcirculatie verminderde. Echter, wanneer de patiënt werd losgekoppeld van de hartlongmachine bleek dat patiënten die aan pulsatiele flow waren blootgesteld een sterke verbetering lieten zien van de doorbloeding van de microcirculatie, terwijl de doorbloeding van de microcirculatie in patiënten die aan een niet-pulsatiele bloedstroom waren blootgesteld onverminderd verslechterd bleef na de operatie. Het verschil in de doorbloeding van de microcirculatie tussen de twee groepen kon niet worden verklaard door verschillen in ontstekingsparameters.
Het gebruik van een pulsatiele bloedstroom resulteerde tevens in een verbetering van het zuurstofmetabolisme ten opzichte van patiënten die aan een niet-pulsatiele bloedstroom waren blootgesteld. Het gunstige effect van een pulsatiele bloedstroom kan hoogstwaarschijnlijk worden toegeschreven aan bescherming van de binnenbekleding van de bloedvaten, en niet aan een direct effect van de puls op de vaatwand omdat het gunstige effect van een pulsatiele bloedstroom pas na afkoppeling van de hartlongmachine zichtbaar werd.

In **Hoofdstuk 5** werden de bloedstroomprofielen in de microcirculatie onder de tong geanalyseerd in patiënten die wel of niet aan de hartlongmachine werden aangesloten tijdens

de hartoperatie. We onderzochten of acute fysiologische veranderingen tijdens het gebruik van de hartlongmachine leiden tot maldistributie van bloed in de microcirculatie. Onze data tonen aan de blootstelling aan de hartlongmachine leidt tot een verhoogde heterogeniteit van de bloedstroom in de microcirculatie, en dit fenomeen is nog steeds aanwezig na de operatie. In een derde van de haarvaten blijken dusdanig hoge bloedstroomsnelheden te ontstaan dat het afgeven van zuurstof aan de weefsels bemoeilijkt wordt. Parallel hieraan ontstaat een suboptimaal zuurstofmetabolisme, wat geassocieerd is met een verminderde zuurstofconsumptie door de weefsels. De bovengenoemde fenomenen waren afwezig in patiënten die een off-pump hartoperatie ondergingen. Uit deze studie concludeerden we dat er een herverdeling van de bloedstroom ontstaat in de microcirculatie wanneer patiënten worden aangesloten op een hartlongmachine. Deze herverdeling gaat gepaard met zeer hoge bloedstroomsnelheden in de microvaten, wat kan leiden tot een verandering in de zuurstofafgifte van bloed aan de weefsels. Een suboptimale zuurstofafgifte kan bijdragen aan het ontstaan van orgaanschade, en zou mogelijk voorkomen kunnen worden door de doorbloeding van de microcirculatie te beschermen en te handhaven tijdens hartoperaties.

In **Hoofdstuk 6** onderzochten we of het gebruik van de hartlongmachine de samenstelling van het bloed dusdanig beïnvloedt dat dit resulteert in veranderingen in de cellen die de binnenbekleding van de vaatwand vormen, de zogenaamde endotheelcellen. De endotheelcellen vormen doorgaans een barrière die het andere cellen en schadelijke stoffen lastig maakt om in de weefsels door te dringen, de zogenaamde endotheliale barrièrefunctie. Hiervoor moeten deze cellen echter sterke verbindingen vormen. De hypothese van het onderzoek was dat blootstelling aan de hartlongmachine leidt tot het vrijkomen van stoffen in het bloed van de patiënt die de barrièrefunctie van het endotheel verstoren.
Omdat dit onderzoek niet in patiënten kon worden uitgevoerd werden de endotheelcellen opgekweekt in het laboratorium. Vervolgens werden de cellen blootgesteld aan bloedplasma van patiënten die een hartoperatie hadden ondergaan. In de celcultuur was het mogelijk om te meten of de cellen nog een goede barrière vormden. Wanneer de cellen werden blootgesteld aan plasma dat voor de operatie was afgenomen veranderde de barrièrefunctie nauwelijks. Echter, wanneer de cellen werden blootgesteld aan plasma dat was afgenomen na het gebruik van de hartlongmachine verloren de cellen hun barrièrefunctie. Het verlies van barrièrefunctie in de celcultuur kon niet worden voorkomen wanneer bloed werd gebruikt van patiënten die aan een pulsatiele bloedstroom waren blootgesteld tijdens het gebruik van de hartlongmachine. Uit dit onderzoek in het laboratorium bleek dat na het gebruik van de hartlongmachine de vingerafdruk van factoren in het bloed dusdanig verandert dat dit invloed heeft op de barrièrefunctie van endotheelcellen. Daarnaast was deze studie een eerste aanwijzing dat het gebruik van de hartlongmachine mogelijk kan leiden tot een verlies van de endotheliale barrièrefunctie.

Hoofdstuk 7 beschrijft de relatie tussen de aanwezigheid van de glycocalyx, een beschermende suikerachtige structuur die zich op de endotheelcellen bevindt, en de doorbloeding van de microcirculatie in patiënten die een hartoperatie ondergaan. De dikte van de glycocalyx werd indirect gemeten met een camera die onder de tong van de patiënten werd geplaatst. Tegelijkertijd werd met deze camera de doorbloeding van de microcirculatie gemeten. In patiënten die tijdens de hartoperatie aan de hartlongmachine werden aangesloten nam de dikte van de glycocalyx af. Opmerkelijk was dat in patiënten die aan een pulsatiele bloedstroom waren blootgesteld tijdens het gebruik van de hartlongmachine de glycocalyx dikte zich herstelde na de hartoperatie. Dit herstel was afwezig in patiënten die aan een niet-pulsatiele bloedstroom waren blootgesteld. In patiënten die een off-pump operatie hadden ondergaan bleef de glycocalyx dikte relatief stabiel gedurende de operatie. Daarnaast vonden we een verband tussen de dikte van de glycocalyx en de doorbloeding van de microcirculatie. Wanneer dikte van de glycocalyx afnam verminderde ook de doorbloeding van de microcirculatie. Deze studie suggereert dat de doorbloeding van de microcirculatie mogelijk wordt beïnvloedt door de integriteit van de glycocalyx, en dat bescherming van de glycocalyx zou kunnen bijdragen aan behoud van de doorbloeding van de microvaten in het lichaam.

In **Hoofdstuk 8** wordt een experimenteel model voor het gebruik van een hartlongmachine in de rat beschreven. Om de microcirculatie te kunnen bestuderen werd de cremaster spier van de rat vrijgeprepareerd, waarna door middel van intravitaal microscopie opnames van de doorbloeding van de microcirculatie konden worden gemaakt. In dit hoofdstuk werd onderzocht of de verdunning van bloed (hemodilutie) zoals plaatsvindt tijdens blootstelling aan de hartlongmachine de belangrijkste reden is voor het verlies aan de doorbloeding van de microcirculatie. Hiervoor werden ratten blootgesteld aan de hartlongmachine, of werd hun bloed verdund zonder aansluiting aan de hartlongmachine. In beide groepen ontstond er een gelijke mate van verdunning van ongeveer 30%. In een controlegroep waarin het bloed van de ratten niet was verdund bleef de doorbloeding van de microcirculatie vrijwel volledig constant over tijd. Ratten die alleen werden blootgesteld aan verdunning toonden een tijdelijk en mild verlies van de doorbloeding van de microcirculatie. Echter, blootstelling aan verdunning ten gevolge van aansluiting aan de hartlongmachine resulteerde in een vermindering van het aantal doorbloedde microvaten van 40%, en slechts in een deel van deze microvaten werd de doorbloeding hersteld na afkoppelen van de hartlongmachine. Het verlies van de doorbloeding van de microcirculatie ging gepaard met stijging van ontstekingsmarkers en markers voor activatie van de endotheelcellen. Daarnaast werd in de groep die blootgesteld was aan de hartlongmachine meer kenmerken van nierschade gemeten. Uit dit onderzoek concludeerden we dat blootstelling aan de hartlongmachine zelf meer invloed heeft op de doorbloeding van de microcirculatie en de functie van vitale organen dan verdunning alleen.

In **Hoofdstuk 9** stond de hypothese centraal dat het verlies van endotheliale barrièrefunctie leidt tot lekkage van vocht uit de bloedvaten, en dat deze zogenoemde vaatlekkage een belangrijke oorzaak is voor het verlies van doorbloeding van de microcirculatie tijdens het gebruik van de hartlongmachine. Om deze hypothese te onderzoeken maakten we gebruik van het geneesmiddel imatinib, dat bekend staat om zijn beschermende werking tegen vaatlekkage in andere ziektebeelden. Ratten werden wel of niet blootgesteld aan imatinib voordat zij aan de hartlongmachine werden aangesloten. Terwijl de doorbloeding van de microcirculatie tijdens het gebruik van de hartlongmachine sterk achteruit ging in ratten die niet aan imatinib waren blootgesteld, bleek imatinib geassocieerd te zijn met behoud van de doorbloeding van de microcirculatie. Parallel hieraan werd in de dieren die niet aan imatinib waren blootgesteld vaatlekkage geconstateerd in de darmen en longen, maar niet in dieren die met imatinib waren behandeld. De bescherming van de microcirculatie in de imatinib groep ging tevens gepaard met bescherming van de nieren en longen, en resulteerde in een verminderde behoefte aan vochttoediening in deze dieren. Deze resultaten suggereren dat het voorkomen van vaatlekkage, zoals optreedt tijdens blootstelling aan de hartlongmachine, gepaard gaat met behoud van de doorbloeding van de microcirculatie, minder vochtbehoefte en bescherming van vitale organen. Deze experimentele data bieden perspectief voor klinische vervolgstudies die zich richten op bescherming van vitale organen in acute situaties, zoals hartoperaties.

In **Hoofdstuk 10** wordt de algemene conclusie van het proefschrift beschreven, en worden de implicaties en limitaties van het proefschrift bediscussieerd. We beschrijven een hypothese over het ontwikkelen van de verminderde doorbloeding en zuurstofgehalte in de microcirculatie na hartchirurgie bestaande uit twee oorzakelijke factoren. De ontstekingsreactie die ontstaat na blootstelling van bloed aan de hartlongmachine leidt tot activatie van de endotheelcellen met vaatlekkage en een verminderde doorbloeding van capillairen als gevolg. De bijkomende verdunning van het bloed ten gevolge van vloeistoftoediening geeft vervolgens een extra vermindering van de zuurstofvoorziening voor de weefsels. Daarnaast werd geconcludeerd dat dysfunctie van de microcirculatie bij hartchirurgie met gebruik van een hartlongmachine voornamelijk toegeschreven kan worden aan vaatlekkage door endotheelcelactivatie ten gevolge van ontsteking. Zowel pulsatiele bloodstroom door de hartlongmachine als het gebruik van imatinib zijn veelbelovende interventies om de doorbloeding van de microcirculatie te verbeteren en mogelijk ook vitale organen te beschermen tegen dysfunctie na hartchirurgie.

Résumé français

Au cours de cette thèse, nous avons eu pour but d'investiguer les mécanismes sur lesquels repose l'altération de la perfusion microcirculatoire durant la chirurgie cardiaque sous circulation extracorporelle (CEC). Par ailleurs, nous avons évalué deux stratégies thérapeutiques destinées à protéger la microcirculation des stimuli délétères induits par la CEC: une modalité de flux pulsatile durant la CEC et la réduction de la fuite vasculaire grâce à la réduction de la perméabilité endothéliale.

Le **chapitre 1** fournit une introduction sur la chirurgie cardiaque et décrit la morbidité postopératoire reliée à l'utilisation d'une CEC. En outre, la microcirculation et sa pertinence pour les praticiens gérant la période péri opératoire sont décrites.

Le **chapitre 2** fournit une revue des altérations qui surviennent dans la microcirculation au cours de la chirurgie cardiaque avec ou sans utilisation de CEC. Les techniques de monitorage de la microcirculation disponibles pour le clinicien sont décrites. De plus, les facteurs qui peuvent contribuer aux perturbations de la perfusion microcirculatoire durant la chirurgie cardiaque, incluant l'hémodilution, l'hypothermie modérée, l'utilisation de la CEC et les flux non pulsés sont discutés.

Dans le **chapitre 3**, nous avons évalué les caractéristiques de la perfusion de la microcirculation linguale au cours de la chirurgie à cœur battant et au cours de la chirurgie cardiaque sous-pompe en utilisant l'imagerie par Sidestream Darkfield (SDF). La perfusion microvasculaire était correctement maintenue au cours et après la chirurgie à cœur battant, alors que les patients ayant subi une chirurgie sous pompe montrait une diminution de 20% de la densité de la perfusion capillaire après le démarrage de la CEC. La perfusion microvasculaire n'avait pas récupéré lors de l'admission en réanimation dans le groupe sous CEC, en dépit de la normalisation de la température et de l'hématocrite. De façon intéressante, nous n'avons trouvé aucune corrélation entre les paramètres de microcirculation et de macrocirculation. Nous avons conclu que la chirurgie à cœur battant était associée à une préservation de la perfusion microcirculatoire, alors que la chirurgie sous pompe conduisait à une réduction de la perfusion capillaire qui ne récupérait pas en postopératoire immédiat.

Dans le **chapitre 4**, nous avons comparé l'utilisation d'un flux pulsé avec un flux conventionnel non pulsé au cours de la CEC chez des patients subissant une chirurgie cardiaque sous pompe dans un essai randomisé en simple aveugle. Il a été démontré que le flux pulsé peut avoir un effet protecteur sur l'endothélium comparativement à un flux non pulsé, et par conséquent nous avons fait l'hypothèse que le flux pulsé en CEC préserve la perfusion capillaire durant

la chirurgie cardiaque. Les effets sur la perfusion capillaire ont été étudiés par imagerie SDF de la muqueuse sublinguale à de multiples reprises au cours de la chirurgie et lors de l'admission en réanimation. Le flux pulsé n'a pas évité la diminution initiale de perfusion capillaire. Cependant, la récupération de la perfusion microvasculaire après sevrage de la CEC a été améliorée dans le groupe avec flux pulsé comparativement aux patients exposés à un flux non pulsé. Aucune différence n'a été observée en terme de paramètres inflammatoires plasmatiques. La consommation systémique d'oxygène et le taux d'extraction d'oxygène ont été améliorés durant la CEC seulement dans le groupe avec flux pulsé, pouvant indiquer une réduction du shunt microvasculaire. L'effet positif du flux pulsé sur la perfusion capillaire après CEC dépend plus probablement d'un effet lié aux cellules endothéliales que d'un effet direct de la pulsatilité, considérant la récupération retardée de la microcirculation en postopératoire.

Dans le **chapitre 5**, les caractéristiques du flux sanguin dans la microcirculation sublinguale durant la chirurgie sous CEC et à cœur battant ont été évaluées. Nous avons investigué l'hypothèse que des altérations physiologiques aigues durant la CEC conduisent à un shunt systémique dans la microcirculation. Nos données ont montré que la CEC est associée à une augmentation de l'hétérogénéité de la perfusion microvasculaire, et que ce phénomène persiste en postopératoire. En outre, un tiers des capillaires ont révélé des vélocités extrêmement élevées des globules rouges, incompatibles avec une oxygénation normale. En parallèle, la différence artério-veineuse en contenu d'oxygène et la consommation systémique en oxygène ont diminué, indiquant la présence d'un shunt sanguin dans la microcirculation systémique durant et après la CEC. Nous n'avons observé aucune hétérogénéité microcirculatoire ou de détournement microvasculaire au cours de la chirurgie sans CEC. De ces découvertes nous concluons que le détournement microvasculaire systémique est présent chez les patients subissant une chirurgie cardiaque sous CEC et que ceci est associé à une extraction réduite de l'oxygène systémique, alors que ce phénomène n'est pas présent au cours de la chirurgie sans CEC.

Dans le **chapitre 6**, nous avons investigué comment la CEC est associée avec une perte de la fonction de barrière endothéliale sur une culture de cellules endothéliales *in vitro* qui était exposée à du plasma de patients subissant une CEC pulsatile ou non pulsatile. La résistance à travers l'endothélium a été évaluée en utilisant la détection de l'impédance électrique du substrat cellulaire dans le but de quantifier la perméabilité de la monocouche de cellules endothéliales. Le plasma prélevé à l'issue de la CEC a conduit à une augmentation de la perméabilité endothéliale comparativement au plasma de base. Ceci en parallèle d'une augmentation des marqueurs d'activation cellulaire endothéliale après la CEC. L'hémodilution n'a pas eu d'effet sur la perméabilité endothéliale, pas plus que l'utilisation d'un flux pulsé

comparativement à un flux non pulsé. Cette investigation démontre que les composants du plasma de patient au décours d'une chirurgie sous CEC induisent une augmentation de la perte fonctionnelle de la barrière endothéliale dans un modèle *in vitro*. Cet effet peut être relié à une augmentation des marqueurs inflammatoires et d'activation endothéliale.

Le **chapitre 7** décrit la relation entre le glycocalix endothélial et la perfusion microcirculatoire durant la chirurgie cardiaque. Chez des patients subissant une chirurgie sans CEC, ou une chirurgie sous CEC avec flux pulsé ou non-pulsé, nous avons investigué les dimensions du glycocalix endothélial en utilisant une technique d'imagerie sublinguale évaluant les dimensions du glycocalix. Dans les deux groupes subissant une chirurgie sous CEC, les dimensions du glycocalix ont diminué après le départ en CEC, alors qu'une récupération après déconnection de la CEC n'a été observée qu'après CEC pulsatile. La chirurgie sans CEC n'a pas été associée à une réduction des dimensions du glycocalix. En outre, nous avons trouvé une corrélation entre les dimensions du glycocalix et la densité de vaisseaux perfusés dans la microcirculation, suggérant que les dimensions réduites du glycocalix endothélial sont associés avec une perfusion capillaire altérée chez les patients subissant une chirurgie cardiaque.

Dans le **chapitre 8**, un modèle de CEC sur le rat avec une préparation du muscle crémaster pour investigation de la microcirculation a été décrit. Le rôle de l'hémodilution dans le développement de l'altération de la microcirculation durant la CEC a été investigué. Les rats ont subi soit une CEC, soit une hémodilution aigue jusqu'à un hématocrite similaire, soit une fausse procédure simulée. La perfusion microcirculatoire fut non altérée dans ce dernier groupe. Le départ en CEC a conduit à une réduction de 40% de capillaires perfusés, et seulement une faible récupération une heure après déconnection de la CEC. L'hémodilution aigue seule a conduit à une diminution mineure et temporaire de la perfusion microvasculaire. La CEC a induit une augmentation de la réponse inflammatoire et de l'activation endothéliale comparativement à l'hémodilution.
En plus la souffrance rénale évaluée histologiquement a été accrue à l'issue de la CEC. Nous avons conclu que l'hémodilution ne peut pas totalement expliquer l'altération de la perfusion microcirculatoire induite par la CEC, et ne constitue seulement qu'un contributeur mineur et temporaire.

Dans le **chapitre 9** nous avons fait l'hypothèse que la fuite vasculaire durant la CEC est un contributeur majeur de la perfusion capillaire perturbée, et que la diminution des fuites vasculaires préserve la perfusion microcirculatoire. Avant la CEC, des rats ont été randomisés entre un traitement par imatinib, connu pour ses effets protecteurs sur la barrière endothéliale dans des conditions septiques, ou un placebo. La perfusion microcirculatoire

du muscle crémaster a montré une diminution significative à l'issue du démarrage en CEC suite au traitement placebo, là où l'imatinib a conduit à une préservation de la perfusion microvasculaire. Parallèlement, la fuite vasculaire a diminué dans de nombreux organes sous imatinib et en parallèle à une réduction des besoins en fluide durant et après la CEC. En outre, les marqueurs de souffrance rénale et pulmonaire étaient plus faibles chez les rats traités par imatinib comparativement à ceux traités par placébo. Ces résultats suggèrent que la fuite vasculaire contribue de façon importante à l'altération de la perfusion microvasculaire durant la CEC, et que la réduction de la dysfonction de la barrière endothéliale améliore la perfusion de la microcirculation, les marqueurs de souffrance d'organes et réduit les besoins en fluide de remplissage.

Dans le **chapitre 10** les principaux résultats de cette thèse sont discutés, de même que les considérations méthodologiques des études effectuées et les possibles directions futures de recherche. Nous proposons un modèle à deux têtes du développement des perturbations aigues de la perfusion microcirculatoire et de l'oxygénation tissulaire défaillante durant la chirurgie cardiaque sous circulation extracorporelle. La réponse inflammatoire générée par l'exposition du sang au circuit extracorporel conduit à l'activation de l'endothélium et à la fuite vasculaire ayant pour résultat la réduction du nombre de capillaires perfusés. L'hémodilution concomitante aggrave en plus l'oxygénation tissulaire par le biais d'un apport réduit en oxygène. Nous concluons que la dysfonction microcirculatoire au cours de la chirurgie cardiaque sous circulation extracorporelle est principalement attribuée à une augmentation de la fuite vasculaire due à une dysfonction inflammatoire de la barrière endothéliale. Le flux pulsé durant la circulation extracorporelle et le traitement par imitanib sont tous les deux des interventions prometteuses pour l'amélioration de la perfusion microcirculatoire et la réduction des dysfonctions aigues d'organes en chirurgie cardiaque sous circulation extracorporelle.

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Biography

Nick Julius Koning was born on the 20th of June, 1988 in Heemskerk, the Netherlands. Following graduation from high school in 2006 at "OSG Piter Jelles" in Leeuwarden (cum laude), he started studying medicine at the VU University Medical Center in Amsterdam.
During his studies, he participated in the Honours Programme and started doing research at the Department of Anesthesiology under the supervision of prof.dr. C. Boer in 2009. In 2010, he performed a five month research internship in France at the Université d'Angers, supervised by prof.dr. C. Baufreton (Department of Cardiovascular Surgery), where the basis for the experimental studies of the current thesis was formed. Nick continued his research as a parttime PhD student besides his medical studies under the shared supervision of professors Boer and Baufreton. He received awards for the best oral presentation at the 2012 scientific meetings of the Dutch Society for Anesthesiology and of the Dutch Society for Microcirculation and Vascular Biology.
He completed his medical studies in 2013 after spending the last four months at the "Département de Réanimation Médicale et Medicine Hyperbare, Centre Hospitalier Universitaire d'Angers" (chair prof.dr. A. Mercat) for a clinical internship as "externe" under guidance of prof.dr. P. Asfar.
In 2014, he started his residency in Anesthesiology at the VU University Medical Center (chair prof.dr. S.A. Loer) and continued his training program at the Spaarne Ziekenhuis in Hoofddorp (supervision Drs. C.W.P. Van der Hoeven and Drs. E.L.V.M.M. Wiewel) in 2015. In 2016, he spent another four months in Angers at the Medical Intensive Care, this time as "interne", as a part of his residency training. He thereafter continued his Anesthesiology formation in Amsterdam.

UNIVERSITE BRETAGNE LOIRE

VU VRIJE UNIVERSITEIT AMSTERDAM

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Thèse de Doctorat

Nick Julius KONING

Protection of the microcirculation during cardiac surgery with cardiopulmonary bypass

Résumé

La chirurgie cardiaque sous circulation extra-corporelle conduit à une altération de la perfusion de la microcirculation, qui peut contribuer de façon importante à la dysfonction d'organe postopératoire. Cette thèse rassemble des études cliniques et animales, dont le but était d'investiguer les mécanismes expliquant la dysfonction microcirculatoire en chirurgie cardiaque sous circulation extra-corporelle. En outre nous avons eu pour but d'évaluer deux stratégies thérapeutiques pour la préservation de la perfusion microcirculatoire au cours de la circulation extra-corporelle : l'utilisation d'un flux pulsé comparativement à un flux non pulsé conventionnel durant la circulation extra-corporelle, et le traitement par imatinib dans le but de réduire la fuite vasculaire en inhibant la dysfonction de la barrière endothéliale.

La thèse actuelle a démontré que la perfusion microcirculatoire est altérée durant et après la chirurgie cardiaque, et que ceci peut être attribué principalement à la dysfonction inflammatoire de la barrière endothéliale et à la fuite vasculaire conséquente. L'hémodilution concomitante en chirurgie cardiaque sous circulation extra-corporelle peut s'ajouter et contribuer également à la réduction de la perfusion microcirculatoire et de l'oxygénation. Nous avons montré que l'utilisation d'un flux pulsé durant la circulation extracorporelle améliore la perfusion microcirculatoire en postopératoire comparativement à un flux non-pulsé. Le traitement par imatinib a réduit la dysfonction de la barrière endothéliale et la fuite vasculaire dans notre modèle de circulation extracorporelle sur le rat et a permis de préserver la perfusion microcirculatoire et l'oxygénation durant et après la circulation extra-corporelle. En outre, le traitement par imatinib a permis de diminuer les marqueurs de souffrance rénale, pulmonaire et digestive après circulation extra-corporelle. A partir de nos résultats, la réduction de la fuite vasculaire et l'utilisation d'un flux pulsé durant la circulation extra-corporelle sont des interventions prometteuses pour la prévention des complications postopératoires chez les patients à risque de défaillance d'organe au décours de la chirurgie cardiaque sous circulation extra-corporelle.

Mots clés

Chirurgie cardiaque, circulation extra-corporelle, endothélium, inflammation, microcirculation.

Abstract

Cardiac surgery with cardiopulmonary bypass leads to impaired perfusion of the microcirculation, which may be an important contributor to postoperative organ dysfunction. This thesis combines clinical and animal studies that aimed to investigate the mechanisms underlying microcirculatory dysfunction in cardiac surgery with cardiopulmonary bypass. Moreover, we aimed to evaluate two treatments strategies for preservation of microcirculatory perfusion during cardiopulmonary bypass: the use of pulsatile flow as compared to the conventional nonpulsatile flow during cardiopulmonary bypass and treatment with imatinib in order to reduce vascular leakage by inhibiting endothelial barrier dysfunction.

The current thesis has demonstrated that microcirculatory perfusion is impaired during and after cardiac surgery, and this can be attributed mainly to inflammatory endothelial barrier dysfunction and consequent vascular leakage. Concomitant hemodilution may additionally contribute to reduced microvascular perfusion and oxygenation in on-pump cardiac surgery. We showed that the use of pulsatile flow during cardiopulmonary bypass improves postoperative microvascular perfusion as compared to nonpulsatile flow. Imatinib treatment reduced endothelial barrier dysfunction and vascular leakage in our rat model for cardiopulmonary bypass and resulted in preservation of microcirculatory perfusion and oxygenation during and after extracorporeal circulation. Moreover, imatinib treatment resulted in reduced markers of renal, pulmonary and intestinal injury after cardiopulmonary bypass. Based on our findings, reduction of vascular leakage and use of pulsatile flow during cardiopulmonary bypass are promising interventions for the prevention of postoperative complications in patients at risk for organ failure following cardiac surgery with cardiopulmonary bypass.

Key Words

Cardiac surgery, cardiopulmonary bypass, endothelium, inflammation, microcirculation.