Connecting hemosysis and visceral injury during cardiovascular surgery : studies on the causes, effects, and treatment of hemolysis-induced organ injury

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Connecting hemolysis and visceral injury during cardiovascular surgery

**Studies on the causes, effects, and treatment
of hemolysis-induced organ injury**

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Connecting hemolysis and visceral injury during cardiovascular surgery

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Ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. L.L.G. Soete,
volgens het besluit van het College van Decanen,
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General introduction

INTRODUCTION

Cardiovascular disease is the major cause of death worldwide, claiming over 7.2 million lives in 2004 due to coronary heart disease alone.¹ In the Netherlands, the total number of patients diagnosed with coronary heart disease was 730.400 in 2007 (RIVM). In 2008, 16.877 patients required open heart surgery to sufficiently treat coronary and/or cardiac valve disease.²

The introduction of the cardiopulmonary bypass (CPB) in the early nineteen sixties as a more routine technique during cardiovascular surgery led to a significant increase in treatment options for patients with serious cardiovascular disease, such as patients with multifocal coronary artery disease, cardiac valve disorders or patients with major vascular disease such as (thoracoabdominal) aortic aneurysms. CPB, also called extracorporeal circulation, enabled ongoing organ perfusion and oxygenation during extensive and time-consuming operations of the heart and/or aorta. Nevertheless, despite major technical and medical refinements since its first introduction, CPB-assisted surgery is still associated with (relatively) high morbidity and mortality. Principally, major postoperative complications such as renal dysfunction (requiring dialysis), and pulmonary complications such as prolonged mechanical ventilation, ventilation assisted pneumonia or the adult respiratory distress syndrome, are relatively common.³⁻⁶ Several CPB-related factors, such as ischemia-reperfusion injury, hemodilution, and initiation of a pro-inflammatory response, have been extensively studied for their role in the development of these postoperative complications.⁷ Furthermore the impact of intravascular hemolysis caused by mechanical stress within the CPB system, among other factors, has been mentioned in the literature but not related to organ injury or patient outcome.

This thesis aimed to elucidate the causes of acute hemolysis and its effects on the development of postoperative complications after (major) cardiovascular surgery.

POSTOPERATIVE COMPLICATIONS OF CARDIOVASCULAR SURGERY WITH CARDIOPULMONARY BYPASS

Open (thoraco)abdominal aortic aneurysm repair

The term *aneurysm* denotes an abnormal dilatation of a blood vessel. Although theoretically any blood vessel may become aneurysmal, the infrarenal abdominal aorta (AAA) is most commonly affected. In contrast, the most extensive type of aneurysmal disease of the large arteries; the thoracoabdominal aortic aneurysm (TAAA) is a less commonly diagnosed phenomenon representing only 0.25% of all aortic aneurysms.⁸ Outward rupture of the weakened vessel wall represents the major risk of any aneurysmal blood vessel. Rupture of an aortic aneurysm is usually fatal due to severe

hemorrhage and, if possible, requires emergency repair with concomitant high surgical morbidity and mortality.⁹ Depending on the type of TAAA, 2-year survival rates of 24-52% have been reported. The overall 5-year survival rate is 17-19%.¹⁰ Aneurysms can be surgically treated either through classical open reconstruction or by endovascular exclusion, depending on aneurysm anatomy, patient characteristics and preference and/or experience of the surgeon and hospital facilities. Despite the fact that elective surgical treatment is often imperative to improve survival, mortality rates remain high after open TAAA surgery; up to 15%.¹¹ The primary causes of in-hospital death are cardiac complications (46%), respiratory failure (15%), sepsis (12%) and coagulopathy (12%).¹² The incidence of nonfatal complications after TAAA repair is accordingly high. Significant post-operative complications occur in 77% of cases; 41% of patients have pulmonary complications, 28% renal complications, 21% liver dysfunction, or gastro-intestinal complications in 7% (such as bowel ischemia, ileus, or peptic ulceration).¹³ Finally, spinal cord-injury is seen in 12% of cases. A combination of these complications is often seen (31%) and, at worst, can progress into multiple organ failure (MOF). Nevertheless, successful open TAAA repair causes the 5-year survival rate to triple or even quadruple to 66-80%.^{11,12}

Cardiac surgery

The incidence of complications after cardiac surgery is largely dependent on the type of surgery performed, with higher incidence rates reported after complex cardiac surgery such as combined bypass and valve surgery, and ascending aortic aneurysm repair.¹⁴ The following incidence rates apply to patients undergoing cardiac surgery with CPB.

Acute kidney injury (AKI) is among the most common and serious postoperative complications with reported incidence rates varying between 5%-30%^{4,6,15-17}, depending on the definition used. AKI-associated morbidity and mortality is high, especially when patients require (transient) dialysis.¹⁸ Considering other visceral complications, a recent report of Rodriguez *et al.* indicated that gastro-intestinal complications occur in approximately 1.2% of patients, with an associated mortality rate of 32%.¹⁴ Of these complications, gastrointestinal bleeding is the most common (33%), followed by mesenteric ischemia (13.1%), pancreatitis (12.8%), cholecystitis (9.3%), peptic ulceration (6%), and diverticulitis (3%). Fulminant liver failure had the lowest incidence (2%) but carried highest mortality (55%).¹⁴ Next to visceral complications, patients undergoing cardiac surgery frequently suffer from pulmonary dysfunction which include a spectrum of clinical symptoms ranging from mild postoperative dyspnea, the need for prolonged mechanical ventilation (>48h), to the adult respiratory distress syndrome (ARDS) in 2% of cases with a 50% mortality rate.¹⁹ Lastly, patients often suffer from the development of the systemic inflammatory

response syndrome (SIRS), as cardiac surgery is associated with a strong systemic inflammatory response.²⁰ At worst, although less frequently reported, SIRS may progress into sepsis and septicemia (3.4%)⁶, and MOF (0.5%).^{6,21}

The pathophysiology of complications after cardiovascular surgery with CPB is complex and has been extensively studied before. Therefore, a complete discussion of every aspect of the pathophysiological mechanisms underlying postoperative complications would be beyond the scope of this thesis. Nevertheless, the next paragraph highlights some important factors which play a role in the development of complications after CPB-assisted surgery.

PATHOPHYSIOLOGY OF COMPLICATIONS AFTER CARDIOVASCULAR SURGERY WITH CARDIOPULMONARY BYPASS: TRADITIONAL CONTRIBUTING FACTORS

The complication rate after cardiac surgery is dependent on many patient-related factors, such as increased age, chronic renal dysfunction, and decreased cardiac output. Furthermore, a number of surgery-related factors such as emergency surgery, use of intra-aortic balloon pump, increased number of blood transfusions, and length of CPB time and aortic cross clamp time have been frequently reported. In general, the CPB is considered to play an important role in the pathophysiology of complications after CPB-assisted surgery by induction of hypoperfusion and ischemia-reperfusion injury, coagulation, inflammation, and hemodilution.²²⁻²⁸ In line, lower morbidity and mortality rates after off-pump coronary artery bypass grafting (OPCAB surgery) have generally been explained by the absence of the CPB.²⁹ Surprisingly however, the largest randomized controlled trial to date on this subject (the ROOBY trial), covering 2203 CABG patients, actually reported on worse composite outcome and poorer graft patency 1 year postoperatively in patients subjected to OPCAB surgery compared with patients in the on-pump group.²¹

Ischemia-reperfusion injury

Ischemia-reperfusion injury describes the seemingly paradoxical injury caused by reperfusion of ischemic tissue.³⁰ Ischemia, either due to insufficient or absent blood flow, deprives downstream tissue cells from oxygen and nutrients, and allows enhanced expression of proinflammatory gene products (cytokines), while more protective gene products such as nitric oxide synthase (which enables nitric oxide formation) are repressed. Furthermore, ischemia stimulates leucocyte attraction. Paradoxically, re-introduction of oxygen enables formation of toxic reactive oxygen species, and triggers an intense inflammatory response, contributing to severe cellular damage in not only the ischemic tissue itself, but also in remote organs.^{30,31} Ischemia-reperfusion injury is now appreciated as a crucial pathophysiological mechanism in the

development of tissue damage in the gut³², kidney³³, liver³⁴, heart³⁵, lungs²³, brain³⁶, and skeletal muscle tissue³⁷. The extent and duration of ischemia-reperfusion injury differs between cardiac and aortic aneurysm surgery. During TAAA surgery, connection of the selective perfusion catheters require clamping and opening of the aorta, causing a short period of absolute ischemia distally of the proximal aortic cross clamp. In AAA patients, in whom a CPB is not used, the extent of ischemia-reperfusion depends on the location of the aortic cross clamp. In general, a moderate degree of ischemia-reperfusion injury affects the lower extremities, although this extent of ischemia-reperfusion injury is generally well tolerated.³⁸ In patients undergoing cardiac surgery, the myocardium suffers absolute ischemia (during coronary reconstruction).³⁹ In contrast, the rest of the body is “only” subjected to relative ischemia-reperfusion injury due to the transition from physiologic to non-physiologic, and back to physiologic tissue perfusion before, during, and after CPB, respectively.⁴⁰

Increased coagulation

The extensive non-physiological surface of the CPB causes profound hemostatic derangements during surgery. Exposure of blood to the circuit immediately causes contact activation (intrinsic pathway) of the clotting system through kallikrein, prekallikrein, high-molecular-kininogen, and factor XII.⁴¹ Subsequently, stimulated by complement activation, activation of the extrinsic pathway results in thrombin and fibrin generation, cross-linking of fibrin, and platelet activation. The extrinsic pathway is also directly activated by cellular debris and release of tissue factor from the surgical field and vessel injury. The consequences of extrinsic pathway activation are counteracted by administration of heparin before initiation of CPB. Next to activation of coagulation, consumption and dilution of coagulation proteins occurs as well, provoking a heightened state of fibrinolysis during CPB, imposing increased risk of hemorrhage.⁴² Finally, activation of coagulation also contributes to the inflammatory response by inducing cytokine/chemokine expression and leukocyte migration. The pro-inflammatory role of thrombin, a central player in coagulation, is particularly well described in this setting.⁴³

Inflammation

Activation of inflammation during cardiovascular surgery with CPB is a complex process and occurs at different sites and different times throughout surgery. Contributing factors include surgical trauma⁴⁴, blood contact with the non-physiological surfaces of the CPB, and ischemia-reperfusion injury.⁴⁵ Also, loss of intestinal barrier function due to increased gut permeability may promote translocation of bacteria-derived endotoxins from the intestinal lumen to the circulation.⁴⁶ The ensuing endotoxemia plays an important role in the induction of the

SIRS, sepsis, and multiple organ failure after surgery.⁴⁷ Together, all these factors trigger the complement system with formation of the highly pro-inflammatory C3a and C5a, promote production of several pro-inflammatory cytokines such as IL-6 and IL-8, oxygen free radicals, and increase the expression of adhesion molecules including E-selectin, P-selectin, integrins, and the intracellular adhesion molecule and vascular cell adhesion molecule. This further stimulates infiltration and activation of polymorphonuclear neutrophils.^{24,45}

Hemodilution

After initiation of CPB, the blood of the patient is immediately diluted with the crystalloid priming volume of the CPB-circuit. Hemodilution facilitates a decrease in blood viscosity so that the blood supply to the organs can be maintained without increasing the arterial pressure. Nevertheless, (excessive) hemodilution causes a significant drop in hematocrit (hemodilutional anemia) which adversely affects the oxygen carrying capacity of blood, compromising tissue oxygenation.²⁷ In line, an inverse dose-dependent relation between hemodilution and postoperative renal injury has been reported.⁴⁸ Also, hemocrit levels below 24% were related to enhanced plasma lactate levels (reflecting inadequate oxygen delivery to cells), renal tubular injury, intestinal mucosal damage, and increased blood transfusions requirements, which further promoted tissue injury.²⁸

It becomes evident that the pathophysiology of tissue injury during cardiovascular surgery (with CPB) is complex and multifactorial.

HEMOLYSIS AND THE INTRAVASCULAR NITRIC OXIDE METABOLISM

Potential causes of hemolysis during cardiovascular surgery

Intravascular rupture of red blood cells (RBCs), or hemolysis, is a well known phenomenon during cardiovascular surgery, and is generally attributed to CPB.^{41, 49-56} Prolonged exposure of red blood cells to the mechanical forces within the circuit (especially shear stress), the non-physiological surfaces, roller pump, and blood-air interfaces substantially impairs RBC deformability, and promotes RBC aggregability.⁴⁹ As normal RBC deformability is imperative for RBCs to pass through the microcirculation, loss of deformability causes entrapment of RBC in the microcirculatory bed. Also, decreased RBC deformability and increased RBC aggregability results in significantly enhanced blood viscosity, which further impairs passage of rigid RBCs through the microcirculation.⁵⁷ When RBC damage finally becomes too extensive, rupture of the cell membrane follows, resulting in release of

intracellular RBC components such as hemoglobin (then referred to as free hemoglobin, fHb) and arginase-1. Beside CPB, we consider the use of autotransfusion devices (cell savers) and transfusion of stored RBCs (packed cells) to also significantly contribute to (sub)lethal RBC damage in this setting. Cell savers expose RBCs to significant (centrifugal) forces, inflicting sublethal RBC damage.⁴⁹ Prolonged storage of blood results in both hemolysis and sublethal damage of red cells.⁵⁸ Subsequent transfusion of stored red blood cell products may cause a direct (transfusion of the fHb-containing storage medium), and delayed (rupture of less viable RBCs) increase of fHb in the patient.⁵⁹

Pathophysiology of hemolysis-induced organ injury

Although the development of red blood cell damage is a well-known phenomenon during cardiac surgery, its role in the development of postoperative complications has not been equally studied. In the last decade however, the number of studies reporting on the adverse effect of hemolysis in patients with *chronic* hemolytic diseases has increased substantially. Furthermore, a (new) pathophysiological mechanism by which hemolysis induces tissue injury and organ compromise has been proposed.⁶⁰

Hemolysis is a hallmark characteristic of several conditions such as malaria, sickle cell disease, paroxysmal nocturnal hemolysis, thalassemia, pyruvate kinase deficiency, and autoimmune hemolytic anemia.⁶¹ Although these various chronic hemolytic diseases each have unique symptoms, all patients suffering from hemolysis experience symptoms ranging from mild muscle and chest pains, dysphagia, fatigue, muscle spasms, erectile dysfunction to severe thrombosis and vaso-occlusion, renal and hepatic failure, stroke, severe anemia, and multiple organ failure.⁶²⁻⁶⁴ The relation between hemolysis and these complications was not evident until the association between increased plasma fHb levels, released from ruptured erythrocytes, and a decreased bioavailability of intravascular nitric oxide (NO) in sickle cell disease was described in 2002.⁶⁰ In their landmark paper, the group of Gladwin demonstrated that intravascular scavenging of endothelial-derived NO by oxygenated fHb was related to decreased NO-bioavailability in plasma, and attenuated forearm blood flow.⁶⁰ Furthermore, the vascular response to intra-arterial infusion of nitric oxide donors was abrogated, indicating NO-scavenging.⁶⁰ Before 2002, the NO-scavenging property of oxygenated hemoglobin molecules was already described (**Reaction 1**), but this phenomenon was not linked to the pathophysiology of symptoms complicating diseases associated with increased levels of oxygenated fHb.^{65,66}

The reaction between oxygenated fHb (oxy fHb) is fast ($6\text{-}8\cdot 10^7$ M/L/sec), irreversible and yields methemoglobin (metHb) and nitrate. Beside oxy fHb, also deoxygenated fHb is able to react with NO, generating iron-nitrosyl-Hb (**reaction 2**).

The reaction speed of reaction 2 is lower compared to that of reaction 1 ($1\cdot 10^7$ M/L/sec). Furthermore, reaction 2 is not irreversible in contrast to the reaction between oxyHb and NO.^{60,61} Therefore, the contribution of oxyfHb to the decrease of intravascular NO during hemolysis is greater than that of deoxyfHb.

NO, a gaseous molecule, is synthesized by nitric oxide synthases (NOS) during the conversion of L-Arginine to L-Citrulline (**Figure 1.1**). NO is the principal endogenous vasodilator and is a central regulator of vascular homeostasis. In vivo, constitutive production of NO by endothelial NO synthase (eNOS), accounts for 25-30% of basal human blood flow.⁶⁷ Next to inducing vasodilation, endothelial-derived NO also attenuates platelet activation and aggregation, intravascular thrombosis, and inflammation (leukocyte migration and adhesion) (**Table 1.1**).^{68,69} A reduction of NO bioavailability by fHb therefore explained (or at least further clarified) a variety of complications seen in patients with chronic hemolytic disease.

Table 1.1 Stimulatory and inhibitory effects of NO in the vasculature

Stimulation	Inhibition
+ Vascular smooth muscle cell relaxation	- Platelet aggregation
+ Vasodilation	- Neutrophil adhesion
+ Vascular smooth muscle cell apoptosis	- Vascular smooth muscle cell proliferation
+ Endothelial cell proliferation	- Vascular smooth muscle cell migration
+ Re-endothelialization	- Endothelial cell apoptosis
	- Matrix formation

The human body harbors an intrinsic defense mechanism to increased levels of fHb. Binding of fHb to the plasma (acute phase) protein haptoglobin (Hp) generates fHb-Hp complexes, which reduces, albeit not abolishes, the NO-scavenging potential of fHb.^{70,71} CD163, a membrane receptor expressed by circulating monocytes and tissue macrophages in the liver and spleen, facilitates endocytosis and clearance of the fHb-Hp complexes.⁷² Clearance of fHb through Hp (although beneficial) thus results in concomitant clearance of Hp during excessive hemolysis, increasing the hosts susceptibility to the adverse effects of fHb. Hemopexin forms a second line of defense by scavenging fHb-derived ferric heme, enabling degradation of ferric heme in the liver. This way, hemopexin attenuates both heme-catalyzed oxidative damage and heme-bound iron loss after hemolysis.^{73,74}

Figure 1.1 Simplified scheme of the pathological effects of hemolysis on the intravascular nitric oxide metabolism. Intravascular hemolysis results in release of arginase-1 and free hemoglobin (striped boxes) into the vascular lumen. Free hemoglobin is principally present in its oxygenated form (oxyfHb), and to a lesser extent as deoxygenated fHb (deoxyfHb). Under physiological circumstances, nitric oxide (NO) is generated during the conversion of L-arginine into citrulline by endothelial nitric oxide synthase (eNOS) in the vascular endothelial cells. Enhanced arginase-1 levels can indirectly result in decreased NO bioavailability through conversion of the substrate L-arginine into ornithine, which does not yield NO. OxyfHb and deoxyfHb directly contribute to a reduction of NO-bioavailability through NO-scavenging. The reaction between oxyfHb and NO is stronger and irreversible compared to the reaction between deoxyfHb and NO, resulting in the formation of methemoglobin and nitrate, and iron-nitrosylHb, respectively. A significant reduction in intravascular NO-bioavailability impairs activation of guanylate cyclase in the vascular smooth muscle cells, an enzyme required for the generation of cyclic guanine monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). A reduction in cGMP concentrations results in reduced smooth muscle cell relaxation, limiting vasodilation. This way, hemolysis contributes to impaired tissue perfusion through a reduction of NO-bioavailability.

Beside fHb, also arginase-1 is released (**Figure 1.1**) from ruptured erythrocytes. This enzyme converts L-arginine, the substrate for NO formation, to ornithine and urea.⁷⁵ A reduction in L-Arginine availability might therefore further reduce NO-bioavailability. Consistent with this concept, a significant decrease of the arginase-ornithine ratio, indicating higher arginase activity, was significantly associated with greater severity of pulmonary hypertension, and increased mortality in sickle cell disease.⁷⁶ In contrast, in a report on liver surgery patients in whom significantly increased arginase-1 levels were found, L-arginine concentrations were not altered.⁷⁷ Also, enhanced arginase-1 levels in patients undergoing open TAAA surgery were not related to changes in the arginine-ornithine ratio, suggesting that the impact of arginase release on the arginine metabolism is not (highly) clinically relevant in this setting (Hanssen SJ; Thesis "Hemolysis, microcirculatory changes and organ injury induced by cardiovascular surgery", 2011). Finally, the availability of intracellular L-arginine is in far excess of the

enzymatic requirements (or capacity) of the NOS enzymes. Therefore, a reduction of L-arginine due to increased arginase might not have a major influence on NO-bioavailability. Despite this surplus of arginine availability however, supplementation of arginine has been shown to increase NO concentrations and promote tissue perfusion in vivo.⁷⁸ This phenomenon is commonly referred to as the arginine paradox. In conclusion, the NO-scavenging effect of fHb significantly reduces NO-bioavailability during hemolysis. The contributing effect of arginase-1, through a reduction of L-arginine, on NO-bioavailability during hemolysis remains to be determined.

CLINICAL CONSEQUENCES OF INTRAVASCULAR HEMOLYSIS IN THE ACUTE SETTING

As described in the previous section, the adverse effect of hemolysis on the intravascular NO-metabolism is more and more appreciated in the setting of chronic hemolysis. In several patient and animal studies, increased fHb has now been directly linked to decreased NO-bioavailability by increased plasma NO-consumption, impaired NO-mediated fore-arm vasodilation, increased systemic, pulmonary, and renal blood pressure (through vasoconstriction), vaso-occlusive pain, increased platelet activation, hemoglobinuria, and impaired renal function.^{60,79-83} The adverse effects of fHb on the NO-metabolism were already observed at fHb concentrations of 6 $\mu\text{mol/l}$.⁶⁰

In contrast to the chronic setting, the clinical consequences of acute hemolysis are less extensively studied. Notwithstanding, several studies previously reported on the adverse effects of hemolysis on renal function in patients undergoing cardiovascular surgery. As fHb was believed to merely be toxic after glomerular filtration, these study focused on (or explained the adverse relation by) the direct nephrotoxicity of fHb. In line, increased fHb levels have been associated with renal injury and increased mortality in children and adults after cardiac surgery.^{52,56} Furthermore, patients displaying serum ferritin levels below the median of the study group (ferritin is the principal heme-derived free iron-sequestering protein) were at increased risk of renal injury compared to patients with ferritin concentrations above the median.⁵¹ In a recent (2007) review by Haasse *et al.* on CPB-associated acute kidney injury, the focus again lay on iron-mediated formation of reactive oxygen species within the renal tubuli, and precipitation of fHb molecules causing tubular obstruction.⁸⁴ In this thesis, we propose that the intravascular NO-scavenging property of fHb is as important, or potentially even more important, to the nephrotoxic effect of fHb (and hemoglobin-derived free heme and free iron) for the development of renal injury during cardiovascular surgery. Furthermore, we also evaluated the role of hemolysis in the induction of intestinal injury in this setting, which has, to our knowledge, not been studied before.

In conclusion, fHb has been identified as an important mediator of microcirculatory dysfunction and tissue injury development in different patients populations. The link between increased plasma fHb concentrations and adverse outcome has been particularly well studied in the setting of chronic hemolysis, and to a lesser extent in patients with acute hemolysis. Although this thesis principally focuses on the potential causes and consequences of acutely increased fHb levels during cardiovascular surgery (with cardiopulmonary bypass), fHb may also play an important mechanistic role in other settings (**Table 1.2**).

Table 1.2 Overview of examples of (clinical) settings in which fHb-induced NO-scavenging has been related to adverse outcome (A), or may underlie increased morbidity and mortality (B).

A Established adverse effect		B Potential adverse effect	
- SC-disease	60, 61, 64, 76, 85-92	- Cardiovascular surgery + CPB	T
- Malaria	93-95	- (Massive) blood transfusions	⁵⁹ , T
- Thalassemia	96	- Transfusions with artificial blood substitutes	S
- PNH	97	- Acute hemolytic transfusion reactions	S
- Pre-eclampsia	98	- Mechanical heart valve-induced hemolysis	S
		- Hereditary spherocytosis	S
		- Hereditary stomatocytosis	S
		- Chemical/infection-induced anemia	S
		- Sports-related hemolysis	S

Abbreviations: PNH, paroxysmal nocturnal hemoglobinuria; SC, sickle-cell; T, discussed in this thesis; S, suggested, but not studied.

This thesis largely focuses on the adverse effects of fHb in the development of (visceral) organ injury. Notwithstanding, cell free myoglobin shares several features with fHb, including its NO-scavenging property.⁹⁹⁻¹⁰¹ It may thus be hypothesized that release of free myoglobin during cardiovascular surgery, as a consequence of myocardial damage and/or skeletal muscle injury, also contributes to intravascular NO-scavenging in this setting. Although of particular interest, an overview on the effects of free myoglobin on the NO-metabolism is beyond the scope of this thesis.

POTENTIAL THERAPEUTIC OPTIONS TO REDUCE THE ADVERSE EFFECTS OF INTRAVASCULAR HEMOLYSIS

The identification of the NO-scavenging property of fHb, and the subsequent adverse effect on plasma NO-bioavailability, microcirculatory perfusion, and tissue integrity, has provided a new therapeutic target to improve patient outcome after (chronic) hemolytic episodes. Attenuation of the unwanted consequences of increased plasma fHb can be achieved through inactivation of fHb or supplementation of NO. A full overview of the (potential) therapeutic options lies outside the scope of this introduction as they are discussed in more detail in Chapter 4 of this thesis. However,

another way to counteract fHb-induced impaired vasodilation (or even vasoconstriction) may be to stimulate NO-independent vasodilation. In fact, an increased vasodilatory response (i.e. increased fore-arm blood flow) to acetylcholine (Ach), a partly NO-independent and endothelium-dependent vasodilator, was observed in patients with sickle-cell disease as compared with normal subjects. This observation may indicate (a compensatory) upregulation of non-NO vasodilators in the hemolysis-induced decreased bioavailability of NO. Ach is the principal neurotransmitter of the autonomic/parasympathic nervous system. It has been recently found that the release of Ach can be stimulated by dietary fat.¹⁰² Enteral high fat feeding stimulates cholecystokinin 1 (CCK-1) receptors in the small intestine and subsequently activates afferent and efferent vagal nerve fibers, ultimately leading to enhanced release of Ach.¹⁰³ Stimulation of Ach-bioavailability not only promotes Ach-induced vasodilation (mediated by NO, prostaglandins and prostacyclins), but was also found to significantly preserve intestinal integrity after intestinal hypoperfusion/ischemia-reperfusion (in a rodent model of hemorrhagic shock), and significantly attenuate the ensuing systemic inflammatory response.^{102, 103} Considering the fact that cardiovascular surgery (with CPB) is associated with the development of (intestinal) tissue injury, the induction of (systemic) inflammation, and, as we propose in this thesis, impaired vasodilation through fHb-induced NO-consumption, administration of high fat enteral nutrition may prove to be an interesting therapeutic intervention in this setting, as we investigated in chapter 9.

AIMS OF THIS THESIS

As outlined in this chapter, we consider acute hemolysis and scavenging of intravascular NO by the release of fHb, to be an important contributing factor in the development of postoperative complications after (major) vascular surgery and complex cardiac surgery.

The aims of this thesis were to subsequently:

1. investigate the development and extent of intestinal mucosal injury and renal tubular damage in patients undergoing aortic or cardiac surgery, with and without CPB (Chapter 2, 3, 6, and 7),
2. study the extent of acute hemolysis during aortic surgery and cardiac surgery with and without CPB, and its effects on the NO-metabolism (Chapter 4, 6, and 7),
3. investigate the relation between acute hemolysis and organ injury in aortic and cardiac surgery (Chapter 4, 6, and 7),
4. investigate the potential contribution of transfusion of stored blood to circulating fHb levels in patients (Chapter 8),
5. study the effect of a potential therapeutic role of enteral high fat feeding in a rodent model of acute hemolysis (Chapter 9).

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Circulating intestinal fatty acid binding protein as an
early marker of intestinal necrosis after aortic surgery;
a prospective observational cohort study

Iris C Vermeulen Windsant*, Femke A Hellenthal*, Joep PM Derikx, Martin H Prins,
Wim A Buurman, Michael J Jacobs, Geert Willem H Schurink

* These authors contributed equally to this manuscript

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ABSTRACT

Objective

To evaluate the usefulness of plasma IFABP levels in the early identification of intestinal necrosis (IN) in patients undergoing different types of aortic surgery.

Summary background data

Intestinal compromise greatly contributes to postoperative adverse outcome. IN is the most detrimental form of intestinal compromise and is notoriously difficult to diagnose. IFABP is a small protein exclusively expressed by mature enterocytes and a promising marker of intestinal damage.

Methods

Plasma IFABP concentrations were measured in blood samples taken perioperatively from 55 patients undergoing open thoracic or thoracoabdominal aneurysm repair (OR-TAA(A)), 25 patients undergoing conventional open abdominal aneurysm repair (OR-AAA) and 16 patients undergoing endovascular aneurysm repair (EVAR). Data were compared with perioperative changes in arterial pH and serum lactate.

Results

IFABP levels increased in all patients undergoing OR-TAA(A) and OR-AAA reaching peak levels shortly after surgery; 281 ± 33 pg/ml to $2,298 \pm 490$ pg/ml, $p < 0.001$, and 187 ± 31 pg/ml to 641 ± 176 pg/ml, $p < 0.05$ respectively. IFABP levels were significantly higher in patients undergoing OR-TAA(A) ($p < 0.001$). IFABP levels in EVAR-patients remained at baseline concentrations throughout the study. Four patients (2 OR-AAA, 2 OR-TAA(A)) developed fatal postoperative intestinal ischemia on day 2 or 3 postoperatively. High levels of plasma IFABP at the end of surgery had 100% sensitivity and 98.1% specificity for the identification of patients developing IN. In OR-AAA patients, arterial pH and lactate were of additional discriminating value. Complete discrimination between patients with and without IN using plasma IFABP could be made on the first postoperative day.

Conclusions

Analysis of plasma IFABP levels is of additional value to other current plasma markers in the diagnosis of IN, and enables early identification of patients with IN after aortic surgery days before clinical diagnosis.

INTRODUCTION

Loss of intestinal wall integrity during various types of surgery greatly contributes to the development of the postoperative systemic inflammatory response syndrome, sepsis, and multiple organ failure.¹⁻³ Patients undergoing aortic surgery are at increased risk for intraoperative mesenterial hypoperfusion and subsequent intestinal damage. This is mainly due to preexistent atherosclerotic disease of the mesenteric vasculature and several intraoperative factors such as aortic cross clamping, and use of extracorporeal circulation. In line, the incidence of postoperative intestinal necrosis (IN), the most deleterious consequence of intestinal hypoperfusion and ischemia/reperfusion, is relatively high in these patients, ranging 1-3% after elective open aortic repair⁴⁻⁶, and up to 10% in case of emergency repair for ruptured aortic aneurysms.⁷ IN is significantly less frequent in patients undergoing endovascular repair.⁸ Nevertheless, improved perioperative patient care has not resulted in a decreased incidence and mortality (70 to 90%⁴) of IN following aortic surgery.

Diagnostic delay is considered to be the primary cause of the high mortality rate associated with IN.⁹ Aspecific and inconsistent clinical symptoms often resemble normal or delayed postoperative course (especially after major aortic surgery), making the diagnosis notoriously challenging. Symptoms include abdominal pain, fever, abdominal distension and (bloody) diarrhea, presenting hours to days following surgery. Furthermore, laboratory parameters associated with histologically proven ischemic colitis, such as lactate, are not specific.¹⁰ Routine colonoscopy following open repair allows diagnosis of ischemic colitis on appearance, but fails to reliably differentiate between superficial and reversible mucosal injury and IN. Furthermore, it is not appropriate for the detection of ischemia of the small intestine.¹¹

Intestinal fatty acid binding protein (IFABP) is a sensitive marker of intestinal mucosal damage and has been proposed as a potential tool for the diagnosis of clinically significant intestinal ischemia.¹² IFABP is a small (15kDa) cytosolic protein exclusively expressed by mature enterocytes, and is rapidly released into the circulation upon enterocyte damage.¹³ In a recently published systematic review, Evenett *et al.* designated IFABP as having potential to improve diagnostic accuracy for the diagnosis of intestinal ischemia.¹⁴ In line, Thuijls *et al.* recently demonstrated that plasma and urine IFABP levels were significantly higher in 22 patients with abdominal complaints at the emergency department suspected of intestinal ischemia who finally developed this disease, compared to 24 patients with other final diagnoses.¹⁵ The current study aims to evaluate the incidence and extent of intestinal damage in patients undergoing open and endovascular repair of aortic aneurysms using plasma IFABP. More importantly, it was studied whether IFABP levels could improve early identification of clinically significant, i.e. transmural, postoperative IN in this high risk patient

population, as this type of IN warrants direct medical and surgical intervention and is a direct cause of high morbidity and mortality.

METHODS

Patients

Three groups of patients undergoing aortic surgery with varying levels of surgical complexity were studied. Fifty five patients undergoing elective open repair of a thoracic or thoracoabdominal aortic aneurysm (OR-TAA(A)) were included. These patients were admitted to the Maastricht University Medical Center or the University Hospital of Aachen, between May 2006 and November 2008. Furthermore, we included twenty five patients undergoing elective open infrarenal or juxtarenal abdominal aortic aneurysm repair (OR-AAA), and sixteen patients undergoing endovascular repair of an abdominal aortic aneurysm (EVAR). The latter two patient groups were included at the Maastricht University Medical Center between January 1st 2007 and January 1st 2008. Patients undergoing emergency aortic aneurysm repair were not included. The study was approved by the Institutional Review Board of both institutes and written informed consent was obtained from every patient prior to surgery. All included patients were subjected to IFABP analysis. Patient characteristics and perioperative clinical data were prospectively collected from the patient's medical record. TAAA type was classified according to Crawford's classification.¹⁶ Preoperative renal function was estimated by calculating the estimated glomerular filtration rate according to the abbreviated Modification of Diet in Renal Disease (MDRD) as recommended by the National Kidney Foundation. Acute kidney injury (AKI) was defined according to the Acute Kidney Injury Network (AKIN) classification, based on perioperative creatinine changes.¹⁷

Surgical procedures

Open thoracoabdominal and thoracic aortic aneurysm repair

The surgical protocol was identical in both institutions and has been described in detail elsewhere.¹⁸ In short, a thoracolaparotomy was performed through the sixth intercostal space (or eighth intercostal space in type V TAAA) to provide access to the aneurysm. After heparinization (0.5 mg/kg), extracorporeal circulation was established using a centrifugal pump with membrane oxygenation (Jostra Rotaflow with Jostra Quadrox, Maquet Cardiopulmonary AG, Hirrlingen, Germany). Depending on aneurysm extent, extracorporeal circulation (ECC) was expanded with a 4-branched tubing system enabling pressure-controlled perfusion of the celiac trunk, superior mesenteric artery, renal arteries, and if necessary intercostal arteries during aortic cross clamping

(ACC) with oxygenated blood. Reimplantation of the inferior mesenteric artery was not performed. Postoperatively, all patients were transported to the Cardiothoracic Intensive Care Unit for postoperative monitoring, circulatory and ventilatory support.

Open repair of an abdominal aortic aneurysm

Open AAA repair was performed via a transperitoneal approach through a midline abdominal incision. Depending on the extent of the aneurysm, the aorta was cross clamped either at infra- or suprarenal level. After bilateral cross clamping of the iliac arteries, the aneurysm sac was opened in a longitudinal direction, intraluminal thrombus was removed and a Dacron tube or bifurcated graft (Gelsoft™ Plus, Vascutek LTD, Terumo, Renfrewshire, Scotland) was proximally and distally anastomosed by means of an in-lay technique. Reimplantation of the inferior mesenteric artery was not performed. Postoperatively, all patients were transported to the recovery room.

Endovascular aortic reconstructive surgery

All EVAR procedures were conducted under general anesthesia. Device selection was made on the basis of anatomic suitability and surgeon preference. Vascular access was achieved via a bilateral incision in the groin. Catheters were threaded through the femoral arteries into the aorta, and a self-expandable bifurcated aorto stent-graft (modular components, Zenith graft, Cook, Bjaeverskov, Denmark) was placed distally of the most distal renal artery. If overstenting of the internal iliac artery was unavoidable, the internal iliac artery was excluded by coiling in a separate procedure weeks prior to surgery. A control angiogram at the end of surgery showed patent mesenteric, renal and internal iliac arteries in all patients. Postoperatively, all patients were transported to the recovery room.

Blood Sampling

Peripheral arterial blood was drawn from the indwelling radial arterial line at preset time-points (**Figure 2.1**). For legibility reasons, time-point T4 (15 minutes after removal of ACC (OR-AAA) or cessation of ECC (OR-TAA(A))) will be indicated as “end of surgery” throughout the manuscript. In case the arterial line was removed prior to the second postoperative day, a venous blood sample was collected by venipuncture. Previous studies did not show a difference between plasma levels of IFABP in peripheral arterial and venous blood (unpublished data). Whole blood was collected in EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ), and immediately put on ice. All samples were centrifuged within 15 minutes after collection (1,500g at 4°C for 15 min), plasma was aliquoted and stored at -80°C until further analysis.

Figure 2.1 Timeline of blood sampling for all patient groups.

Overview of blood sample moments (indicated by the black arrows) in the three included patient populations during the study period. For all OR-TAAA and OR-AAA patients: T1, preoperatively; T7, first postoperative day; and T8, second postoperative day. For OR-TAAA patients specifically: T2, just before start of extracorporeal circulation; T3, just before cessation of extracorporeal circulation; T4, 15 minutes after cessation of extracorporeal circulation (reperfusion); T5, 2 hours after reperfusion; T6, 6 hours after reperfusion. For OR-AAA patients: T2, just before aortic cross clamping (ACC); T3, just before removal of the ACC; T4, 15 minutes after removal of ACC (reperfusion); T5, 2 hours after reperfusion; T6, 6 hours after reperfusion; For EVAR-patients: T1, preoperatively; T2, during insertion of guide wires; T3, deployment of stent; T4, 2 hours after stent deployment at recovery; T5, first postoperative day, T6, second postoperative day.

Abbreviations: OR-TAA(A), open repair of a thoracic or thoracoabdominal aortic aneurysm; OR-AAA, open repair of an abdominal aortic aneurysm; EVAR, endovascular aortic aneurysm reconstruction.

Assay for intestinal fatty acid binding protein

After inclusion of all patients, plasma concentrations of IFABP were analyzed in all blood samples to assess intestinal epithelial mucosal injury. A commercially available enzyme linked immunosorbent assay [ELISA] for detection of human IFABP (Hycult Biotech, Uden, the Netherlands) was used according to manufacture guidelines. All samples were determined in duplicate (<10% variation between duplicates was accepted) and averaged for statistical analysis. The detection limit of the ELISA was 41 pg/ml. The IFABP analysis was performed at the laboratory of the department of Surgery at the Maastricht University Medical Centre. A golden standard for the diagnosis of IN is currently lacking, and suspected IN is traditionally confirmed by emergency laparotomy.¹⁹ IFABP levels were quantified prior to retrospective comparison with postoperative clinical data.

Conventional markers for intestinal ischemia: arterial pH and serum lactate

Arterial pH and serum lactate were routinely analyzed by the department of Clinical

Chemistry of the Maastricht University Medical Center or University Hospital of Aachen in the context of standard care. We collected these data to enable comparison to plasma IFABP levels on the following time-points; preoperatively, at the end of surgery, and on the first and second postoperative day. In EVAR patients arterial pH and lactate were not determined.

Statistical analysis

Continuous data in Tables are presented as median (interquartile range; IQR ranging the 25th-75th percentile), and as N (%) for dichotomous data. Continuous data are presented as mean $\pm$ S.E.M (**Figure 2.2**), or as Tukey box-whisker plots (**Figure 2.3**) indicating median, 1st and 3rd quartile (box), highest and lowest value within 1.5 interquartile range (IQR, whiskers), and values outside 1.5IQR (outliers). Differences in patient characteristics between study groups were compared using Pearson Chi-square test for dichotomous variables, with Fisher's correction when appropriate. Continuous data were analyzed using independent sample t-test or Mann-Whitney U-test depending on Gaussian distribution (checked using histograms and normal Q-Q plots). Continuous data with multiple time-points were analyzed using the repeated measures ANOVA with Bonferroni post-hoc correction, or the Friedman test with post-hoc Dunn's correction, depending on Gaussian distribution.

In **Figure 2.2** and **Figure 2.3** we did not analyze the statistical changes of plasma IFABP, arterial pH, or serum lactate in patients with IN due to limited group size. Receiver Operating Characteristic (ROC) curves were drawn by plotting sensitivity against 100%-specificity in order to find the optimal cut-off point of plasma IFABP, arterial pH or serum lactate which most accurately discriminated between patients with and without postoperative IN. The cut-off point was defined as the maximum sum of sensitivity and specificity. Lastly, positive and negative likelihood ratios were calculated. Statistical calculations were made using SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL), and Prism 4.03 for Windows (GraphPad Software Inc. San Diego, CA). A p-value < 0.05 was considered to indicate statistical significance. This study adheres to the STANDards for Reporting of Diagnostic Accuracy (STARD) criteria for studies reporting on diagnostic accuracy.²⁰

RESULTS

Patient characteristics and clinical outcome

Ninety six patients undergoing surgical repair of an aortic aneurysm were studied; 55 patients undergoing open thoracoabdominal aortic aneurysm surgery (OR-TAA(A) group), and 41 patients diagnosed with an isolated abdominal aortic aneurysm, of

whom 25 were treated using the conventional open procedure (OR-AAA group) and 16 by endovascular exclusion (EVAR group). Clinical baseline characteristics are presented in **Table 2.1** and Supplemental **Table 2.1S**. OR-TAA(A) patients were significantly younger compared to both OR-AAA and EVAR patients (66 (55-71) vs. 70 (67-76), and 70 (65-77) years, $p < 0.001$). Also, the male-female distribution was statistically significantly different in the OR-TAA(A) group with 56% ($N = 31$) male patients versus an almost completely male patient population in the other two groups ($p < 0.001$). As expected, intraoperative and postoperative factors associated with surgical complexity were significantly higher in TAAA patients (**Table 2.1**).

Table 2.1 Baseline characteristics, intraoperative variables, and outcome of OR-TAA(A), OR-AAA, and EVAR patients.

Variable	OR-TAA(A) (N = 55)	OR-AAA (N = 25)	EVAR (N = 16)	p-value
<i>Preoperative</i>				
Age (years), median (IQR 25-75%)	66 (55-71)	70 (67-76)	70 (65-77)	<0.001
Male, % (n)	56 (31)	92 (23)	100 (16)	<0.001
<i>Intraoperative</i>				
Aortic cross clamp infrarenal, % (n)	n.a.	80 (20)	n.a.	-
Aortic cross clamp time (min), median (IQR 25-75%)	103 (73-141)	49 (37-60)	n.a.	<0.001
Extracorporeal circulation time (min), median (IQR 25-75%)	154 (107-197)	n.a.	n.a.	-
Blood loss (L), median (IQR 25-75%)	8.9 (5.7-19.5)	1.2 (0.7-2.0)	0.3 (0.2-0.7)	<0.001
Packed cell transfusion (units), median (IQR 25-75%)	10 (7-13)	0 (0)	0 (0)	<0.001
Duration of surgery (min), median (IQR 25-75%)	390 (328-448)	157 (135-199)	90 (75-120)	<0.001
<i>Postoperative</i>				
In-hospital mortality, % (n)	15 (8)	8 (2)	0 (0)	0.24
ICU admission time (days), median (IQR 25-75%)	5 (3-9)	0 (0-1)	0 (0-0)	< 0.001
Duration of hospitalization (days), median (IQR 25-75%)	17 (13-29)	10 (9-16)	5 (4-5)	<0.001

Abbreviations: OR-TAA(A), open repair of a thoracic or thoracoabdominal aortic aneurysm; OR-AAA, open repair of an abdominal aortic aneurysm; EVAR, endovascular aneurysm repair, IQR, 25-75th interquartile range; n.a, not applicable; ICU, intensive care unit.

Four patients (4.2%, 2 OR-TAA(A) and 2 OR-AAA patients) developed lethal postoperative IN as confirmed by emergency laparotomy and histopathological analysis on the second or third postoperative day. In two patients it concerned small IN, in the other two the descending colon and sigmoid were affected. In all patients the inferior mesenteric artery was already occluded prior to surgery. None of the EVAR patients showed clinical signs of IN. Besides the two patients with IN, no other OR-AAA patient died (total in-hospital mortality 8%). In-hospital mortality for OR-TAA(A) patients was 15% ($N = 8$), and 0% in the EVAR group. Others causes of death in the OR-TAA(A) group were sepsis and multiple organ failure (kidneys and lungs) in 4 patients (who died 7, 9, 13, and 14 days postoperatively), severe cerebral infarction in 1 patient, and hemorrhagic shock in 1 patient.

Supplemental Table 2.15 Preoperative risk factors and outcome in OR-TAA(A), OR-AAA, and EVAR patients.

Variable	OR-TAA(A) (N = 55)	OR-AAA (N = 25)	EVAR (N = 16)	p-value
<i>Preoperative</i>				
PAOD, % (n)	13 (7)	44 (11)	19 (3)	0.005
Diabetes type II, % (n)	6 (3)	4 (1)	6 (1)	0.24
Hypertension, % (n)	70 (35)	44 (11)	44 (7)	0.07
Smoking, % (n)	45 (23)	20 (5)	31 (5)	0.05
COPD, % (n)	18 (10)	20 (5)	31 (5)	0.52
Stroke, % (n)	4 (2)	25 (4)	6 (1)	0.07
Previous coronary artery bypass grafting, % (n)	13 (4)	22 (2)	13 (2)	0.26
Preoperative renal function (GFR, ml/min/1.73m ²), median (IQR 25-75%)	74 (58-93)	62 (57-77)	56 (51-88)	0.15
Preoperative chronic renal dysfunction (GFR<60), % (n)	28 (15)	29 (5)	50 (8)	<0.001
Maximum aneurysm diameter (mm), median (IQR 25-75%)	68 (56-77)	64 (59-76)	63 (58-68)	0.75
<i>Aneurysm extent</i>				
TAAA type (according to the Crawford classification)				
Type I, % (n)	9 (5)	n.a.	n.a.	-
Type II, % (n)	20 (11)	n.a.	n.a.	-
Type III, % (n)	42 (23)	n.a.	n.a.	-
Type IV, % (n)	16 (9)	n.a.	n.a.	-
TAA, % (n)	13 (7)	n.a.	n.a.	-
Infrarenal abdominal aortic aneurysm, % (n)	n.a.	80 (20)	94 (15)	0.09
<i>Postoperative</i>				
Uncomplicated postoperative course, % (n)	40 (21)	55 (10)	88 (14)	0.003
Postoperative intestinal necrosis, % (n)	4 (2)	8 (2)	0 (0)	0.62
Dialysis, % (n)	11 (6)	6 (1)	0 (0)	0.55

Abbreviations: OR-TAA(A), open repair of a thoracic or thoracoabdominal aortic aneurysm; OR-AAA, open repair of an abdominal aortic aneurysm; EVAR, endovascular aneurysm repair, IQR, 25-75th interquartile range; n.a., not applicable; PAOD, peripheral arterial occlusive disease; COPD, chronic obstructive pulmonary disease; CVA, cerebrovascular accident; GFR, glomerular filtration rate.

Open aortic aneurysm repair is associated with intestinal epithelial cell damage

To study intestinal epithelial cell damage during surgery we analyzed plasma IFABP in all patient samples (**Figure 2.2**). Preoperative IFABP levels were similar between OR-TAA(A), OR-AAA, and EVAR patients without postoperative IN (**Figures 2.2A, 2.2C, and 2.2E**) and with postoperative IN (**Figures 2.2B and 2.2D**). During surgery, IFABP levels increased in all patients undergoing open aortic aneurysm repair reaching peak levels at the end of surgery (281 ± 33 pg/ml to $2,298 \pm 490$ pg/ml, $p < 0.001$, and 187 ± 31 pg/ml to 641 ± 176 pg/ml, $p < 0.05$ for OR-TAA(A) and OR-AAA patients respectively (**Figures 2.2A and 2.2C**). The pattern of IFABP change during the study period was similar in OR-TAA(A) and OR-AAA patients. Nevertheless, peak IFABP concentrations were statistically significantly higher in the first group ($p < 0.001$ and $p < 0.005$, at the end of surgery and two hours after removal of the ACC / cessation of ECC, respectively). IFABP levels in EVAR-patients remained at baseline concentrations throughout the perioperative period ($p > 0.05$, **Figure 2.2E**).

Figure 2.2 Plasma IFABP levels in patients undergoing aortic aneurysm repair with and without postoperative intestinal necrosis.

IFABP levels (mean $\pm$ SEM) in patients undergoing open thoracoabdominal aortic aneurysm repair (2.2A/B), open abdominal aortic aneurysm repair (2C/D), and endovascular abdominal aortic aneurysm repair (2.2E). Figures 2.2B and 2.2D depict the two OR-TAA(A) and two OR-AAA patients who developed postoperative IN, respectively. These patients displayed significantly higher IFABP levels compared to patients without postoperative IN. Furthermore, plasma IFABP levels remained significantly increased at the first postoperative day. The dotted line is set at the upper reference limit of all patients (815 pg/ml). The black arrows indicate the difference between the high IFABP levels in patients with IN compared to patients without IN.

Abbreviations: IFABP, intestinal fatty acid binding protein; OR-TAA(A), open repair of a thoracic or thoracoabdominal aortic aneurysm; OR-AAA, open repair of an abdominal aortic aneurysm; EVAR, endovascular aortic aneurysm reconstruction; EVAR, endovascular aortic aneurysm reconstruction; IN, intestinal necrosis; ACC, aortic cross clamp; min, minutes; ECC, extracorporeal circulation.

As IFABP is renally filtered, we analyzed whether differences in preoperative renal clearance might have accounted for differences in IFABP levels between OR-TAA(A), OR-AAA and EVAR patients. Glomerular filtration rate did not correlate to IFABP levels during surgery; $R_s = 0.11$ ($p = 0.31$) and $R_s = 0.006$ ($p = 0.96$) for IFABP levels at the end of surgery and two hours after removal of the ACC / cessation of ECC, respectively (data not shown). In all patients without IN, plasma IFABP values returned to baseline levels on the first postoperative day. When comparing IFABP levels between patients with and without IN, two results stand out. First, peak IFABP levels were markedly higher in patients with postoperative IN already during surgery, and second, plasma IFABP concentrations remained high on the first postoperative day in patients with IN.

Plasma IFABP discriminates patients with and without intestinal necrosis at the end of surgery and on the first postoperative day

The differences in plasma IFABP values between patients with and without postoperative IN already at the end of surgery, while IN was only *clinically* suspected two days later, substantiates its potential as an early biomarker of IN. Consequently, we compared the predictive power of plasma IFABP with two biochemical parameters commonly used in the diagnosis of intestinal ischemia: arterial pH and serum lactate. Although their sensitivity and specificity for IN is debated^{21,22}, the lack of more specific biochemical markers still often compels clinicians to rely on these markers in clinical practice. In general, open aortic surgery was associated with a significant drop of arterial pH and increase in serum lactate (**Figure 2.3**), and these changes were more marked in patients undergoing OR-TAA(A). OR-AAA patients that later developed postoperative IN displayed significantly lower arterial pH at the end of surgery and on the first postoperative day compared to patients without postoperative IN ($p < 0.05$, **Figure 2.3C**). This in contrast to OR-TAA(A) patients, in whom arterial pH levels were not statistically different between patients with and without IN on all studied time-points (**Figure 2.3A**). Serum lactate concentrations were not statistically higher in patients with IN compared to patients without IN in both patient groups on all time-points (**Figures 2.3B** and **2.3D**).

Subsequently, we assessed ideal cut-off points and predictive value of each marker at their peak level at the end of surgery and on the first postoperative day (**Tables 2.2A and 2.2B**). Both arterial pH and serum lactate levels at the end of OR-AAA surgery allowed significant discrimination between patients with and without postoperative IN ($p = 0.02$, and $p = 0.04$ at the end of surgery and on the first postoperative day, respectively). Arterial pH still displayed significant discriminative power on the first day after surgery in this patient group ($p = 0.04$). In contrast, the discriminative power of arterial pH and lactate was markedly lower, and not statistically significant, in OR-TAA(A) patients on both time-points. Lactate levels in OR-AAA patients on the first

postoperative day were also non-discriminative. Lastly, we studied the discriminative power of plasma IFABP levels on the same time-points. Based on these data, detection of plasma IFABP levels above the cut-off points of 5,787 pg/ml (OR-TAA(A) patients) or 1,938 pg/ml (OR-AAA patients) at the end of surgery enabled good discrimination of patients with and without postoperative IN. Interestingly, plasma IFABP concentrations on the first postoperative day were able to discriminate patients with and without IN with 100% sensitivity and 100% specificity in both groups when IFABP levels exceeded 3,646 pg/ml or 1,121 pg/ml for OR-TAA(A) and OR-AAA patients respectively (**Tables 2.2A** and **2.2B**). Summarizing; plasma IFABP has clear (additional) predictive value for detection of patients with postoperative IN after aortic surgery.

Table 2.2 Statistical accuracy of arterial pH, serum lactate and plasma IFABP levels at the end of surgery and on the first postoperative day for the identification of patients with and without postoperative intestinal necrosis.

A. OR-TAA(A) patients						
	<i>End of surgery</i>			<i>1st postoperative day</i>		
	Arterial pH	Plasma lactate	Plasma IFABP	Arterial pH	Plasma lactate	Plasma IFABP
Cut-off value	< 7.245	> 5.3 mmol/l	> 5,787 pg/ml	< 7.255	> 7.2 mmol/l	> 3,646 pg/ml
Area under ROC-curve	0.76	0.86	0.98	0.87	0.58	1.00
P-value	0.22	0.09	0.02	0.08	0.72	0.02
Sensitivity (%)	100	100	100	100	50	100
Specificity (%)	61.2	77.6	98.1	87.0	97.8	100
Likelihood ratio +	2.6	4.5	52.6	7.7	45.5	∞
Likelihood ratio -	0	0	0	0	0.5	0

B. OR-AAA patients						
	<i>End of surgery</i>			<i>1st postoperative day</i>		
	Arterial pH	Plasma lactate	Plasma IFABP	Arterial pH	Plasma lactate	Plasma IFABP
Cut-off value	< 7.215	> 3.8 mmol/l	> 1,938 pg/ml	< 7.255	> 3.6 mmol/l	> 1,121 pg/ml
Area under ROC-curve	1.00	0.97	0.98	0.95	0.69	1.00
P-value	0.02	0.04	0.03	0.04	0.41	0.02
Sensitivity (%)	100	100	100	100	50	100
Specificity (%)	100	93.8	95.7	87.5	91.7	100
Likelihood ratio +	∞	16.1	23.3	8	12.0	∞
Likelihood ratio -	0	0	0	0	0.5	0

Abbreviations: ROC, receiver operating curve; OR-TAA(A), open repair of a thoracic or thoracoabdominal aortic aneurysm; OR-AAA, open repair of an abdominal aortic aneurysm.

Figure 2.3 Change of arterial pH and serum lactate in patients undergoing open aortic surgery with and without postoperative intestinal necrosis.

Arterial pH and serum lactate was routinely measured preoperatively, at peak IFABP levels at the end of surgery, and on the first and second postoperative day. Data are presented using Tukey box-whisker plots indicating median, 1st and 3rd quartile (box), highest and lowest value within 1.5 interquartile range (IQR, whiskers), and values outside 1.5IQR (outliers). Patients with IN are presented separately ($\triangle$ for OR-TAA(A) patients and $\square$ for OR-AAA patients). Figure 2.3A and 2.3B depict the change of arterial pH and serum lactate for OR-TAA(A) patients. Figure 2.3C and 2.3D for OR-AAA patients, respectively.

Abbreviations: IFABP, intestinal fatty acid binding protein; OR-TAA(A), open repair of a thoracic or thoracoabdominal aortic aneurysm; OR-AAA, open repair of an abdominal aortic aneurysm; IN, intestinal necrosis; ACC, aortic cross clamp; min, minutes; ECC, extracorporeal circulation; **/** $p < 0.01$ and $p < 0.001$ compared to preoperative values of group without IN; # $p < 0.05$ between groups (patients with IN versus patients without IN).

DISCUSSION

Intraoperative intestinal damage and intestinal barrier dysfunction is implicated as an important inciting event in the development of postoperative complications.²³ Indeed, loss of viable enterocytes and increased intestinal permeability result in enhanced systemic levels of pro-inflammatory cytokines and endotoxin; factors associated with adverse outcome.^{24,25} Transmural IN is the most deleterious form of intestinal damage and is related to significant morbidity and mortality, mainly attributed to diagnostic delay. Intestinal fatty acid binding protein is a promising marker of intestinal compromise in man.^{12,26} The aim of the current study was to investigate whether plasma IFABP enables timely identification of patients that develop clinically significant transmural IN after aortic surgery. To that end, the kinetic properties of plasma IFABP were studied in patients undergoing three types of aortic surgery. Furthermore, we compared the predictive value of IFABP levels with two clinically used biochemical markers for IN; arterial pH and serum lactate.

In this study we showed a transient but significant increase in IFABP levels in all patients undergoing open aortic aneurysm surgery, indicating the development of intestinal damage. Moreover, we found higher IFABP concentrations in patients undergoing OR-TAA(A) compared to patients undergoing OR-AAA. The development of intestinal damage during aortic surgery can result from a temporary low-flow state of the mesenteric and hypogastric circulation due to anesthesia and hypovolemia, and from intestinal macro-infarction due to embolization of cholesterol plaques or intraluminal thrombus.^{5,26,27} Three specific factors may explain the differences in IFABP levels between OR-TAA(A) and OR-AAA patients. First, patients undergoing OR-TAA(A) experience a brief period of absolute intestinal ischemia when the extracorporeal circulation system and selective organ perfusion catheters are connected, causing profound ischemia-reperfusion injury to the entire intestinal epithelial lining. In contrast, patients undergoing OR-AAA do not experience a period of absolute intestinal ischemia, although (infrarenal) aortic cross clamping has been related to blood flow perturbations of the intestinal tract.²⁸ Second, OR-TAA(A) is associated with significant intraoperative hemolysis due to the extracorporeal circulation, cell saver use and massive blood transfusion.²⁹ Subsequent release of free hemoglobin causes intravascular nitric oxide scavenging which may result in organ perfusion perturbations and subsequent tissue injury.³⁰ This way, hemolysis might significantly contribute to increased IFABP levels in OR-TAA(A) patients. To our knowledge, OR-AAA surgery is not associated with hemolysis. Third, splanchnic hypoperfusion during OR-TAA(A) is further aggravated by the extracorporeal circulation system itself.³¹ These intraoperative differences between the two patient groups may be responsible for more extensive intestinal injury in patients subjected to OR-TAA(A) surgery, thus resulting in higher

cut-off values of IFABP for detection of postoperative IN compared to OR-AAA patients. Nevertheless, the cut-off value of IFABP of OR-AAA patients on the first postoperative day also enabled full discrimination of OR-TAA(A) patients with and without IN.

Another key finding of this study was that patients with postoperative IN displayed exceptionally high IFABP levels at the end of surgery, and on the first postoperative day. In line, plasma IFABP concentrations carried significant prognostic power for postoperative IN on both time-points. Since plasma IFABP is a new marker of IN in this setting, we compared the predictive power of plasma IFABP levels to arterial pH and serum lactate concentrations. Interestingly, both arterial pH and serum lactate levels at the end surgery displayed similar predictive power to plasma IFABP for the identification of patients with IN in OR-AAA patients. This observation is in line with the fact that aberrant arterial pH and lactate values often reflect tissue hypoxia in situations such as lower limb ischemia due to aortic cross clamping, intestinal necrosis, the acute compartment syndrome, ischemic neurological injury, systemic hypoperfusion, shock, SIRS, and sepsis.³²⁻³⁵ In contrast to OR-AAA patients, arterial pH and lactate displayed only limited, and not significant, predictive power after OR-TAA(A). We attribute this to the complex pathophysiological condition of patients after OR-TAA(A). Nevertheless, the combination of the three parameters can provide the clinician with important information on intestinal cellular compromise. The decrease in arterial pH and simultaneous increase in lactate point to cellular hypoxia, however of unspecific origin. Additionally enhanced plasma IFABP values (as seen in our patients with IN) specifically indicate intestinal epithelial cell damage, making it very likely that the changes in arterial pH and lactate are a direct consequence of cellular hypoxemia of the intestinal mucosa.

Our results indicate that plasma IFABP could be a valuable (additional) marker for the early identification of patients with IN after aortic surgery. Ideally, measurement of plasma IFABP should not only serve as a handle in the identification of patients with IN, but also in the initiation of therapeutic measures to minimize further deterioration of intestinal integrity. As IFABP values were already discriminative at the end of surgery, thorough assessment of end-of-surgery bowel viability is imperative. Unfortunately, no sign of impaired bowel viability such as discoloration, absence of peristalsis or mesenteric pulsations, or presence of mucosal hemorrhage, are conclusive for the presence of irreversible IN at that early time-point.³⁶ This implies that identification and resection of the (potentially) affected intestinal segment at the end of surgery will be challenging, if not impossible. Nevertheless, intraoperative IFABP concentrations can be used to identify patients who warrant additional surgical and clinical attention. Therapeutic interventions should be aimed at optimizing intestinal perfusion and prevent bacterial translocation for instance through reimplantation of the inferior

mesenteric artery (although heavily debated), intra-arterial infusion of thrombolytic agents, strict (hemodynamic) monitoring, volume resuscitation, and administration of broad-spectrum antibiotics.^{37,38} Alfa adrenergic agents must be used cautiously since they can cause additional splanchnic vasoconstriction in high doses.³⁹ Enteral feeding might increase intestinal metabolic demand and should be given conservatively until gut viability is confirmed.⁴⁰ A second-look laparotomy or laparoscopy within 24-48 hours remains the golden standard to establish the full extent of non-viable bowel in patients suspected of IN.³⁶ However, selection of patients who would benefit from such an invasive procedure should be done cautiously as surgical re-exploration exposes patients to additional surgical morbidity. Currently, selection of these patients is challenging due to unreliable biochemical and clinical signs and symptoms. Our results indicate that IFABP levels on the first postoperative day are useful to identify those patients who would benefit from an early relaparotomy or relaparoscopy. In short; plasma IFABP based identification of patients with postoperative IN can be done 24-48 hours earlier than clinical selection criteria would currently allow. This is of great importance as early surgical intervention is associated with improved outcome in these patients.⁴¹

A last interesting result from this study was that EVAR patients did not show any sign of intestinal cellular compromise. Also, none of the patients developed clinical postoperative IN. The absence of intestinal damage in EVAR patients is in line with the clinical observation that IN is less frequent in EVAR patients versus those undergoing open aortic repair.⁸ The absence of increased IFABP levels in EVAR patients may indicate a considerable role for intestinal manipulation in the release of IFABP during surgery. However, absence of intestinal manipulation in 20 children undergoing major spinal surgery was still accompanied by increased IFABP levels during surgery.²⁶ Micro and macro embolization has been suggested to be the predominant factor in the development of intestinal injury during EVAR.²⁷ We did not investigate the formation of (micro) emboli and can therefore not relate such emboli with the absence of enhanced IFABP levels. As significant blood loss is considered to hamper intestinal perfusion, the significantly lesser degree of blood loss during EVAR may favor optimal intestinal perfusion during surgery as compared to OR-AAA and OR-TAAA(A) patients. Furthermore, the absence of intestinal mucosal injury could have (in part) accounted for the overall improved postoperative outcome of patients undergoing EVAR surgery as IFABP levels have been shown to correlate with postoperative IL-6 and IL-8 concentrations.²⁴ However, due to the limited number of EVAR patients, we can not substantiate this hypothesis statistically. Paradoxically we observed a higher incidence of preoperative chronic renal dysfunction in patients undergoing EVAR compared to open aortic surgery. Although this may indicate a selection bias, we have as yet no clear explanation for this result.

Summarizing, this study offers two clinically significant findings. First, assessment of plasma IFABP levels enables early identification of patients developing IN after open aortic repair, irrespective of the type of surgery. Second, patients undergoing EVAR do not display detectable intestinal injury. As our study included only a relatively small number of patients with IN (although with a high number of negative controls), our results preclude definitive conclusions. Nevertheless, the current results encourage further study into the predictive value of plasma IFABP, especially combined with arterial pH and serum lactate levels, for the early identification of patients with postoperative IN. In conclusion, we consider assessment of plasma IFABP to be important in the early identification of patients with lethal postoperative IN, thus enabling reduction of the current surgical delay in the treatment of IN and improving patient outcome after aortic surgery.

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Visceral injury and systemic inflammation
in patients undergoing extracorporeal circulation
during aortic surgery

Sebastiaan J Hanssen, Joep P Derikx, Iris C Vermeulen Windsant, John H Heijmans,
Thomas A Koeppel, Geert W Schurink, Wim A Buurman, Michael J Jacobs

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ABSTRACT

Objectives

Visceral injury and inflammation are evaluated in patients undergoing extracorporeal circulation (ECC) either with distal aortic perfusion (DAP) during thoracic aortic aneurysm (TAA) repair or DAP and selective organ perfusion (DAP & SP) during thoracoabdominal aortic aneurysm (TAAA) repair.

Summary background data

Visceral hypoperfusion and subsequent visceral injury, mainly to the gut, have been implicated as central events in the development of systemic inflammatory response syndrome (SIRS) and organ dysfunction after major surgery. Patients undergoing DAP or DAP & SP are exposed to artificial visceral perfusion, potentially leading to the development of intestinal injury and systemic inflammation.

Methods

To assess visceral injury arteriovenous differences of Fatty Acid Binding Proteins were measured for the gut (IFABP and LFABP) and left kidney (LFABP) along with systemic plasma concentrations. Systemic ALT was used as liver injury marker. Plasma IL-6 and IL-8 denoted systemic inflammation.

Results

During ECC systemic IFABP and LFABP levels increased in both groups, representing intestinal injury. Significantly elevated levels of IFABP ($p < 0.001$) and LFABP ($p < 0.001$) were found in the DAP & SP group, after ECC was stopped and normal circulation restored. Liver and renal tubular cell injury was not detected. Significant increases in systemic IL-6 and IL-8 values were measured only in patients undergoing DAP & SP. Additionally, the extent of intestinal injury correlated positively with systemic inflammation.

Conclusions

This study shows the development of intestinal mucosal injury during ECC with DAP or DAP & SP, indicative of insufficient intestinal perfusion. Intestinal injury was associated with a systemic pro-inflammatory response.

INTRODUCTION

Open surgical repair of thoracic aortic aneurysms (TAA) and thoracoabdominal aortic aneurysms (TAAA) are extensive, complex procedures that are associated with significant post-operative morbidity and mortality. Post-operative complications include respiratory failure, renal and liver dysfunction, spinal cord injury, and gastrointestinal complications.¹⁻³

Experimental studies of major surgery and trauma suggest that intestinal injury and hypoperfusion of visceral organs lead to initiation of a systemic pro-inflammatory response.^{4,5} Therefore, visceral protection during aortic surgery is warranted.^{6,7}

The extracorporeal circulation (ECC) technique has been implemented in open TAA repair to provide (retrograde) distal aortic perfusion (DAP) via the femoral artery. In case of TAAA repair involving the origin of visceral arteries, ECC with DAP is combined with selective organ perfusion catheters (DAP & SP) to provide designated visceral arteries directly with blood.^{8,9} The rationale behind DAP and DAP & SP is to reduce ischemic injury of visceral organs, in particular the highly susceptible intestinal mucosa.¹⁰

The advantages of visceral protection notwithstanding, ECC during cardiac surgery provides antegrade aortic perfusion of visceral organs and is still associated with loss of intestinal barrier function seen as endotoxin translocation, indicating intestinal mucosal injury.¹¹⁻¹³ The development of intestinal organ injury during antegrade aortic perfusion is strongly associated with gastrointestinal complications and mortality.^{14,15} Moreover, recent publications suggest ECC with antegrade aortic flow to result in a poorly understood state of 'functional' hypoperfusion of visceral organs, which is related to visceral injury and the induction of SIRS.^{14,16,17}

The quantification of (subclinical) visceral injury during open TAA / TAAA repair could provide valuable information on the perfusion adequacy of ECC with either DAP or DAP & SP. Therefore, in this study we measured intestinal and renal release of fatty acid binding proteins, small cytosolic proteins that leak from injured cells into the circulation, and systemic plasma alanine aminotransferase (ALT) to evaluate liver injury. The pro-inflammatory cytokines interleukin (IL)-6 and IL-8 were measured to assess systemic inflammation. The aim of the present study was to investigate the development of visceral injury in patients undergoing either DAP or DAP & SP, and to evaluate the relation of visceral injury to the systemic inflammatory response in these patients.

METHODS

Patient population

Patients that underwent elective open TAA or TAAA repair between January 2006 and April 2007 at the University Hospital Maastricht or the University Hospital Aachen were included in the study. The study was approved by the local Institutional Review Board of both institutes, and written informed consent was obtained from every patient. Pre-operative clinical data were prospectively collected from the patient's medical record. Crawford's classification was used to specify TAAA type.¹⁸

Surgical procedure

In all patients the same anesthetic protocol was used. The following anesthetic and surgical procedure has been described previously in detail.^{9,19} Collapse of the left lung in all patients except type IV TAAA was enabled by either a double-lumen endotracheal tube or a selective left main bronchial blocker. Induction of anesthesia was achieved with etomidate and sufentanil, and maintained with sufentanil and ketamine. All patients were placed in a left helical position, and thoracotomy or thoracolaparotomy through the sixth intercostal space (eighth intercostal in patients with TAAA type IV aneurysms) was performed.

After heparinization, (0.5 mg/kg), for all patients ECC with DAP was established after cannulation of the left pulmonary vein and the femoral artery, connected to a centrifugal pump. Alternatively, the femoral vein was cannulated with a long cannula placed in the vena cava inferior close to the right atrium; in all cases an oxygenator was part of the ECC. For DAP & SP, a 4-branched tubing system was connected to the ECC and 4 catheters with inflatable tips and flow probes were used for selective perfusion of the celiac axis, the superior mesenteric artery and both renal arteries.

Typically, the aortic reconstruction was done from proximal to distal. During proximal cross-clamping, transection of the aorta, and completion of the proximal anastomosis, DAP was maintained at a mean pressure of 60 mmHg or higher. After abdominal aortotomy, all four selective perfusion catheters were installed in patients undergoing TAAA repair. In ten patients (N = 10) we evaluated directly after insertion the mean blood flow through each individual SP catheter (**Table 3.1**). In general, the celiac axis, superior mesenteric artery and the right renal artery were anastomosed simultaneously as an island in the aortic graft. Just before finishing the anastomosis, the perfusion catheters were removed, indicating that ischemic time is limited to the timeframes of insertion and removal of the catheters. In most cases, the left renal artery was treated with a selective graft using selective perfusion during end-to-end anastomosis. After completion of the anastomosis, the tip of the catheter was

withdrawn into the graft, and perfusion continued, until implantation into the aortic tube graft was accomplished.

Table 3.1 Characteristics of patients undergoing open TAA / TAAA repair

	ECC with DAP (N = 8)	ECC with DAP & SP (N = 22)	P-value
Total procedures (N)	8	22	
TAA	8	-	
TAAA [*]	-	22	
TAAA II	-	3	
TAAA III	-	9	
TAAA IV	-	10	
Male (N)	4	11	1.000
Dissection	4	2	0.033
Mean age (yr)	60.0 (3.4)	63.5 (2.7)	0.483
BMI ^{**} (kg/m ²)	26.9 (1.2)	23.9 (0.8)	0.058
Diabetes	-	2	1.000
Hypertension	5	17	0.643
Smoked	4	19	0.395
Hypercholesterolemia	1	11	0.099
Chronic renal failure	-	2	1.000
CAD [^]	1	2	1.000
COPD ^{^^}	-	5	0.280
ACC [#] time (min)	86 (9)	86 (7)	0.986
ECC time (min)	131 (36)	142 (9)	0.677
Flow SP catheter [†] (mL/min)			
Celiac axis	-	202.0 (33.2)	
Superior mesenteric artery	-	311.6 (30.4)	
Left renal artery	-	212.6 (33.3)	
Right renal artery	-	186.4 (27.7)	
Total operation time (min)	326 (17)	399 (15)	0.015

^{*} Classification according to Crawford.¹⁸ ^{**} BMI, body mass index; [^] CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease. [#] ACC, aorta cross clamp; Continues variables are expressed as mean (SEM). [†] Flow in SP catheter was measured in ten (N = 10) patients undergoing ECC with DAP & SP.

Blood sampling

Peripheral arterial blood was drawn at set time-points (**Figure 3.1**) from the radial artery pre-operatively (Pre-op), while creating access to the aorta, but before clamping the aorta and ECC was initiated (Cannulation), during ECC just before ending DAP or DAP & SP (ECC), 15 minutes after the proximal aortic cross clamp was removed and reperfusion (R) initiated (15 min R), at 2 hours, 6 hours and 10 hours in the reperfusion phase (2 hrs R, 6 hrs R and 10 hrs R), as well as at day 1 and 2 postoperatively (Day 1 and Day 2). Whole blood was collected in EDTA vacutainers (Becton Dickinson, Franklin

Lakes, NJ) and immediately put on ice. Blood was centrifuged and plasma was stored in aliquots at - 20°C until further analysis.

To enable venous blood sampling from the intestines and left kidney in eight patients (N = 8) undergoing DAP & SP, a 16 G single lumen central venous catheter kit was used to cannulate the portal vein (Terumo Corporation, Tokyo, Japan), and a 18 G intravenous catheter (Venflon, Becton Dickinson, Franklin Lakes, NJ) was used to cannulate the left renal vein prior to starting ECC. This enabled us to sample specific arterial and venous blood from the gut and the left kidney in order to calculate organ release and consumption of plasma proteins. Blood was taken from these cannulas before ECC, during ECC with DAP & SP when a sample was taken from the arterial line of the ECC as well, and at 15 minutes reperfusion (**Figure 3.1**).

Figure 3.1 Timeline of blood sampling. At given time-points blood was drawn from the radial artery line and arterial line of the extracorporeal circulation (ECC). Patients undergoing open TAA repair received ECC with distal aortic perfusion (DAP – group), while ECC with DAP and selective organ perfusion was used in patients undergoing open TAAA repair (DAP & SP – group). In order to enable simultaneous arterial and venous organ blood sampling, in eight patients receiving ECC with DAP & SP, a venous catheter was inserted in the portal vein, as well as the left renal vein. This allowed specific calculation of arteriovenous concentration differences for the gut as well as the left kidney. Abbreviations: TAA, Thoracic Aortic Aneurysm; TAAA, Thoracoabdominal Aortic Aneurysm; ECC, extracorporeal circulation; DAP, distal aortic perfusion; SP, selective organ perfusion R, Reperfusion; RA, radial artery; PV, portal vein; RV, renal vein; aECC, arterial line of ECC.

Measurements

Visceral organ injury

Fatty acid binding proteins (FABP) are small cytosolic proteins (15 kDa) that are abundantly expressed in visceral tissue and can be used as sensitive plasma markers to detect early cellular injury.^{20–22} Two types of FABP, intestinal-type FABP (IFABP) and

liver-type (LFABP), are expressed at the tips of the villi of intestinal mucosal epithelial cells. IFABP is only present in intestinal mucosal cells; in contrast, LFABP is also expressed in hepatocytes, and to a lesser extent in renal tubular cells, and increased plasma levels could therefore represent hepatocyte or renal tubular cell injury as well.²³ In order to evaluate hepatocellular injury systemic common liver injury marker ALT was measured. Furthermore, to study renal tubular cell injury as cause of elevated circulating LFABP levels, the arteriovenous concentration difference for the left kidney ($[\text{arterial concentration}] - [\text{venous concentration}] / [\text{arterial concentration}] \times 100\%$) of LFABP was calculated in eight patients (N = 8) undergoing DAP & SP.

Plasma IFABP and LFABP levels were analyzed using Enzyme Linked Immuno Sorbent Assays (ELISA). These commercially available ELISA kits were kindly provided by Hycult biotechnology (Uden, the Netherlands) and were used according to manufacture guidelines. The detection limit was 41 pg/ml for IFABP and 102 pg/ml for LFABP.

Systemic inflammatory response

The plasma levels of the cytokines IL-6 and IL-8 were assayed to determine the systemic inflammatory response following TAA / TAAA repair. IL-6 and IL-8 are commonly used as general inflammatory markers, amongst others produced by stimulated monocytes and activated endothelium, and considered key players in the development of SIRS after major trauma and surgical stress.^{17,24,25}

IL-6 and IL-8 ELISA techniques have been described elsewhere.²⁶ Briefly, 96-well plates (Greiner Bio-One, Kremsmünster, Austria) were coated overnight (4°C) with the appropriate antibodies. Monoclonal antibodies 5E1 and 3E3 were used as coating for IL-6 and IL-8, respectively. Free sites were blocked with 1% bovine serum albumin in PBS. Samples and standard dilution series were incubated for 2 hours. Human recombinant IL-6 and IL-8 were used for standard titration curves. Washing buffer contained 0.1% Tween-20. Biotinylated anti-human IL-6 and IL-8 were developed in our own laboratory and used as detection antibodies. Finally, 3,3', 5,5'-tetramethylbenzidine (TMB) was used as a substrate and the reaction was stopped by adding 1M H₂SO₄. Plasma samples were analyzed in the same run spectrophotometrically (450 nm) using an automated ELISA reader. The detection limits for the assays were 12.3 pg/ml (IL-6) and 46.0 pg/ml (IL-8).

Statistical analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM). Kolmogorov-Smirnov test was used to test for Gaussian distribution. Student's T-test for independent-samples and Chi-square test, followed by Fisher's exact test when needed, were used to test differences between pre-operative patient characteristics. Changes in parameters over time were tested using repeated measures ANOVA,

followed by paired Student's T-tests versus Pre-op values with post-hoc Bonferroni correction, if significant differences were found. In the same way changes in arteriovenous concentrations differences of plasma proteins were tested, with values measured during cannulation as reference. To characterize the total amount of intestinal injury and systemic inflammation during the study period, area under the curve (AUC) for IFABP and LFABP, as well as IL-6 and IL-8 was calculated for each patient. To assess association between intestinal mucosal cell injury and inflammation Spearman correlation analysis between AUC_{IFABP} , AUC_{LFABP} , AUC_{IL-6} , and AUC_{IL-8} was performed. Mann Whitney U test was used to calculate differences in AUC for each parameter between both patient groups. Statistical calculations were made using SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL), and GraphPad Prism software (GraphPad Software, Inc. San Diego, CA). A p-value < 0.05 was considered to indicate statistical significance.

RESULTS

Patient characteristics and intra-operative data

Thirty consecutive patients that underwent TAA (N = 8) or TAAA (N = 22) repair were included in the study. **Table 3.1** shows pre-operative clinical data of patients undergoing ECC with DAP (TAA patients) or DAP & SP (TAAA patients). No significant differences in pre-existing co-morbidities were present in the two patient groups studied, except for the co-existence of an aortic dissection (DAP N = 4 / 8 versus DAP & SP N = 2 / 22, p = 0.033). Nevertheless, aortic dissection did not influence the surgical approach to repair the aneurysm.

Aorta cross-clamp time (ACC), total ECC time and total operation time were noted during all procedures. ACC and total ECC time did not differ significantly between both groups. However, a significant difference in total operation time was found, reflecting the operative complexity of patients undergoing TAAA repair (DAP 326 (17) minutes, and DAP & SP 399 (15) minutes, p = 0.015).

Extracorporeal circulation and intestinal mucosal injury

Pre-operative IFABP levels (DAP 326.8 (84.8) pg/ml, and DAP & SP 411.7 (82.4) pg/ml, **Figure 3.2A**) were comparable to the values measured in a healthy population,²⁷ demonstrating the absence of intestinal mucosal cell injury. Manipulation of visceral organs to create access to the aorta, did not lead to elevated circulating IFABP levels. In contrast, during ECC IFABP levels increased in both groups representing the development of intestinal mucosal injury which was supported by statistically significant repeated measures ANOVA in both groups (DAP, p < 0.05; DAP & SP,

$p < 0.0001$, data not shown). Shortly after artificial circulation was terminated and normal circulation was restored, plasma IFABP levels increased further (DAP 763.9 (200.7) pg/ml, $p > 0.05$; DAP & SP 2351.0 (378.1) pg/ml, $p < 0.001$) and remained elevated for six hours. The release of IFABP in patients undergoing DAP & SP was clearly more pronounced than in patients undergoing DAP, indicating more intestinal injury in the DAP & SP group. Total AUC of systemically measured IFABP was calculated for both groups and revealed significantly more IFABP release in the DAP & SP patient group ($p < 0.05$, data not shown).

Intestinal mucosal cell injury leads next to IFABP release to LFABP release, although, plasma LFABP increase can also be caused by liver and renal tubular cell injury. LFABP plasma levels showed a similar pattern as I-FABP, potentially supporting intestinal injury (**Figure 3.2B**). The total calculated AUC_{LFABP} was significantly higher in patients undergoing DAP & SP ($p < 0.01$, data not shown).

Figure 3.2 Elevated plasma IFABP and LFABP levels in patients undergoing DAP or DAP & SP.

(A) Highest values for IFABP, reflecting intestinal injury, were found during reperfusion for both groups. (B) Systemic plasma LFABP values measured in both patient groups mimicked IFABP release, potentially representing intestinal injury. Values are presented as mean $\pm$ SEM and are compared to pre-operative values. Data were analyzed using paired Student's T-tests with post-hoc Bonferroni correction. * $p < 0.01$; ** $p < 0.001$

Abbreviations: DAP, distal aortic perfusion; SP, selective perfusion; I-FABP, intestinal fatty acid binding protein; ECC, extracorporeal circulation; min, minutes; hrs, hours.

To ascertain the intestinal origin of the released IFABP and LFABP during and shortly after ECC, the arteriovenous concentration differences were measured in eight patients ($N = 8$) undergoing DAP & SP (**Figure 3.3A**). In line with the systemic measurements, before starting ECC there was no fractional increase in IFABP indicating the absence of IFABP release (+ 0.3 (1.8) %). However, during ECC with DAP & SP a significant release of IFABP was found (+ 53.5 (11.2) %, $p < 0.001$), which still occurred at 15 minutes after ECC had been stopped (+ 28.0 (3.3) %, $p < 0.05$). Similar to the IFABP release reported above, no detectable LFABP release from the gut was found before starting ECC with DAP & SP (+ 1.9 (2.8) %). During ECC with DAP & SP, LFABP

release from the intestinal mucosa increased significantly (+ 103.0 (17.5) %, $p < 0.001$) and release of LFABP, specifically indicating intestinal injury, was still statistically significant shortly after ECC perfusion was terminated and normal circulation was restored (+ 51.5 (13.9) %, $p < 0.05$).

Figure 3.3 Intestinal IFABP and intestinal LFABP release was evaluated using arterio-venous concentration differences in eight patients undergoing ECC with DAP & SP. Arterial blood from the radial artery (or the arterial line of the ECC) and the portal vein were simultaneously sampled and intestinal I-FABP and L-FABP release was calculated using the following formula: venous concentration minus arterial concentration divided by arterial concentration $\times 100\%$. (A) Significant release of IFABP and LFABP from the gut was observed during ECC and directly in the reperfusion phase when normal visceral circulation was restored. (B) Circulating concentrations ALT, a specific liver enzyme was measured pre-operatively, at 2 hours reperfusion and at day 1 postoperatively. ALT levels showed no significant increase at the given time points, indicating no significant liver injury. Values are shown as mean $\pm$ SEM and compared to pre-operative values (or values found during cannulation) using paired Student's T-tests with post-hoc Bonferroni correction. * $p < 0.05$; ** $p < 0.001$. Abbreviations: DAP, distal aortic perfusion; SP, selective perfusion; I-FABP, intestinal fatty acid binding protein; L-FABP, liver fatty acid binding protein; ALT, alanine transaminase; ECC, extracorporeal circulation; min, minutes; hrs, hours.

Since LFABP is next to the gut also expressed in liver and kidneys, we studied hepatocellular and renal tubular cell injury. To exclude liver injury, ALT was measured at three different time-points (**Figure 3.3B**); ALT, a cytosolic enzyme mainly expressed in the liver, was not elevated in the early reperfusion phase or at post-operative day 1. These data support the intestinal origin of LFABP release during open TAA / TAAA repair.

Potentially, renal tubular cell injury also results in elevated plasma LFABP concentrations. Therefore, to evaluate release from injured renal tubular cells, we measured arteriovenous concentration differences of LFABP for the left kidney from the same eight patients as reported above. The calculated mean arteriovenous concentration difference before going on ECC was - 21.5 (4.0)%. During ECC with DAP & SP (- 27.6 (6.0)%) and after ECC, when the patients were on normal visceral circulation again (- 22.8 (4.4)%), arteriovenous concentration differences were not

significantly altered compared to values before ECC, indicating an absence of release from injured renal tubular cells (data not shown).

To analyze correlations between intestinal organ injury markers IFABP and LFABP, the total AUC_{IFABP} and AUC_{LFABP} , of all patients undergoing ECC ($N = 30$) were correlated (**Figure 3.5**). A strong positive correlation was found between AUC_{IFABP} and AUC_{LFABP} ($r = 0.70$, $p < 0.0001$).

Taken together, the circulating plasma levels of both I-FABP and L-FABP were elevated during ECC and a further increase was observed in the early phase of restored normal circulation in patients undergoing open TAA / TAAA repair. This indicates intestinal injury, with release of these proteins being more prominent when ECC with DAP & SP was used.

Systemic inflammatory response and extracorporeal circulation during TAA / TAAA repair

Circulating pro-inflammatory cytokines IL-6 and IL-8 were measured in order to evaluate the inflammatory response associated with TAA / TAAA repair. Plasma concentrations of IL-6 before ECC started were not significantly different from baseline values (**Figure 3.4A**). Although changes in plasma IL-6 levels were observed in both patient groups, the mean IL-6 values measured in the DAP patients group were not statistically significantly altered during the studied period (ANOVA $p > 0.05$, data not shown). During visceral perfusion using ECC combined with DAP & SP plasma IL-6 levels increased significantly, indicating the development of a systemic inflammatory response (ANOVA $p < 0.0001$, data not shown). Release of IL-6 continued until peak-values were reached 2 hours after reperfusion (DAP & SP 295.7 (62.7) pg/ml, $p < 0.001$), whereas after 6 hours reperfusion and later plasma values lowered, but remained elevated until day 2 post-operative.

The pro-inflammatory cytokine IL-8, a strong neutrophil attractant, was detectable in pre-operatively taken blood samples from five out of twenty-two patients who underwent DAP & SP (**Figure 3.4B**). Initially, plasma IL-8 curves mimicked IL-6 kinetics, starting to increase during the reperfusion phase in both groups. However, further increase in plasma IL-8 levels in patients who underwent ECC with DAP was relatively small (ANOVA $p > 0.05$, data not shown), whereas increase in plasma IL-8 was significant in patients who underwent ECC with DAP & SP (ANOVA $p < 0.0001$, data not shown), with peak values found at 2 hours reperfusion (227.4 (43.5) pg/ml, $p < 0.05$), indicating a strong pro-inflammatory, chemotactic response in these patients. At postoperative day 2, plasma IL-8 had almost disappeared, being non-detectable in patients who underwent DAP, and only detectable in plasma from four out of twenty-two patients who underwent DAP & SP.

Figure 3.4 The systemic inflammatory response is initiated during ECC.

(A) Increased levels of the pro-inflammatory cytokine IL-6 are found in patients receiving DAP (TAA patients) or DAP & SP (TAAA patients) during artificial visceral perfusion using ECC. Peak values were reached at 2 hours reperfusion (2hrs R). (B) Marked IL-8 release in patients receiving DAP & SP. After initial release during ECC combined with DAP & SP, peak plasma values for patients undergoing TAAA repair were found at 2 hours reperfusion (2hrs R). Values are mean $\pm$ SEM and compared to pre-operative values using paired Student's T-tests with post-hoc Bonferroni correction. * $p < 0.05$; ** $p < 0.001$. Abbreviations: DAP, distal aortic perfusion; SP, selective perfusion; I-FABP, intestinal fatty acid binding protein; L-FABP, liver fatty acid binding protein; ECC, extracorporeal circulation; min, minutes; hrs, hours.

The increase of IL-6 and IL-8 observed during TAA / TAAA repair, indicates the development of a systemic inflammatory response after DAP or DAP & SP; IL-8 release was significant in patients undergoing DAP & SP only, signifying a strong systemic neutrophil attractive response in this patient group.

Figure 3.5 Association between intestinal mucosal cell injury and systemic inflammation in patients undergoing open TAA / TAAA repair. Patient's total AUCs of I-FABP, L-FABP, IL-6 and IL-8 were measured from pre-operative up until day 2 postoperative. Data are matched observations, and degree of correlation is represented by Spearman R. Visceral injury, denoted by AUC_{I-FABP} , strongly correlated with AUC_{L-FABP} , AUC_{IL-6} , and AUC_{IL-8} . These data suggest intestinal injury to play a significant role in systemic inflammation in patients undergoing open TAA / TAAA repair. Abbreviations: I-FABP, intestinal fatty acid binding protein; L-FABP, liver fatty acid binding protein; IL, interleukin; AUC, area under the curve; min, minutes; hrs, hours.

Intestinal injury and systemic inflammation in patients undergoing extracorporeal circulation

To investigate whether intestinal injury was related to the systemic inflammatory response, correlations of AUC_{I-FABP} with AUC_{IL-6} and AUC_{IL-8} (**Figure 3.5**) were analyzed in all patients undergoing ECC (N = 30). AUC_{I-FABP} correlated significantly with both AUC_{IL-6} ($R_s = 0.64$, $p < 0.001$) and AUC_{IL-8} ($R_s = 0.54$, $p < 0.01$), indicating an association between the extent of intestinal organ injury and systemic inflammation, underlining the need for intestinal organ protection during TAA / TAAA repair.

DISCUSSION

In this study intestinal mucosal cell injury, but not hepatic or renal tubular cell injury, occurred in patients undergoing elective open TAA or TAAA repair, despite artificial visceral perfusion using ECC with either DAP (TAA patients) or ECC with DAP & SP (TAAA patients). The extent of intestinal injury and pro-inflammatory reaction was

more pronounced in patients undergoing DAP & SP. Furthermore, intestinal injury correlated positively with markers of systemic inflammation.

Visceral perfusion by means of ECC is used to ensure volume and pressure controlled blood flow to abdominal organs, preventing the deleterious effects of intestinal injury.³ ECC with antegrade aortic perfusion is a well-known technique used in cardiac surgery and acknowledged to induce intestinal injury.¹¹ Holmes et al. showed elevated urine IFABP levels following CABG surgery and suggested that intestinal mucosal injury plays a role in inducing a complicated post-operative course.¹⁴ The use of ECC with either DAP or DAP & SP in patients undergoing open TAA / TAAA repair was first described in the 1980s, and these techniques have since been further refined.^{28,29} We described our first experiences with ECC and DAP or DAP & SP in the mid-90s, and both were considered safe techniques to prevent postoperative renal injury, assessed by urine output and serum creatinine following TAA / TAAA repair, and possibly intestinal injury, by subjective observation of the intestines and intestinal motility peri- and post-operatively.^{8,19}

In the present study, the development of injury to visceral organs was investigated using measurements of plasma IFABP and LFABP for intestinal, ALT for hepatic, and LFABP for renal tubular injury. Our data show development of intestinal injury during artificial perfusion of the viscera using ECC, and shortly thereafter when normal perfusion to the viscera was restored. ECC with DAP caused elevated plasma levels of IFABP and LFABP, and ECC with DAP & SP led to significantly elevated circulating IFABP and LFABP levels, despite the intended protective effect of artificial visceral perfusion in patients undergoing open TAA / TAAA repair. The arteriovenous concentration differences over the intestines demonstrate significant release of IFABP and LFABP in patients undergoing ECC with DAP & SP. During reperfusion the systemic plasma concentration of both IFABP and LFABP initially further increased, suggesting either continued wash-out of these proteins from the injured gut mucosa or sustained hypoperfusion injury leading to continued enhanced release of both proteins. These data are in line with low flow states in patients with hypovolemic or cardiogenic shock and inherent blood flow redistribution towards the heart and central nervous system at the expense of visceral organs. The intestinal mucosal epithelial cells are due to the specific anatomy of the small intestinal villus more susceptible to visceral hypoperfusion than hepatocytes or renal epithelial cells. In particular the cells at the tip of the villus, which contain IFABP and LFABP, are due to the sympathetic innervation of the arterioles and vascular anatomy of the villus, most vulnerable to hypoperfusion.^{10,11}

Total intestinal injury seen during ECC was assessed by AUC_{IFABP} , and correlated positively with the AUC_{IL-6} and AUC_{IL-8} , suggesting an association between the extent of intestinal mucosal injury and the extent of systemic inflammation. Morariu *et al.*

studied the alleged protective use of dexamethasone on the inflammatory response and intestinal injury after CABG surgery with the use of antegrade aortic perfusion.¹⁷ Even though the inflammatory response was attenuated by dexamethasone, no effect on intestinal injury markers was seen, hereby suggesting the inflammatory response to be a result and not so much a cause of intestinal injury.

Pharmacological approaches to reduce visceral hypoperfusion and its sequelae can be directed at either improvement of perfusion or reduction of ischemic injury. Increasing microcirculatory blood flow, especially in the intestines, by pharmacological intervention (e.g. ACE-inhibitors, ET-receptor antagonists or catecholamine therapy) has been shown beneficial in animal studies.³⁰⁻³² Furthermore, also antioxidant therapy (e.g. nitroxide, ascorbic acid or mannitol) has been shown effective in reducing ischemic injury to the intestines in animals.^{33,34} Future studies are needed to evaluate such interventions in the human setting.

The differences in extent of intestinal injury that was observed between DAP during open TAA repair and DAP & SP during open TAAA repair is not easily clarified. Aortic cross clamp times or total perfusion times were similar between the groups. The most likely explanation for the difference in intestinal injury is a more prominent hypoperfusion of the intestines during ECC with DAP & SP. Idu *et al.* showed in a pig model a significant decrease in blood flow per minute to the cranial mesenteric artery when clamping the aorta and perfusion was taken over with ECC and selective organ perfusion.³⁵ Also, Leijdekkers *et al.* calculated that the flow to visceral organs during ECC with DAP & SP is limited and insufficient to maintain tissue perfusion due to the internal diameter in SP catheters used in patients undergoing open TAAA repair.³⁶ The flow administered through the selective organ perfusion catheter system during aortic clamping may be too low in our patients, creating an absolute state of 'functional' hypoperfusion to the intestines. It is of interest, however, that during the reperfusion phase especially in the DAP & SP group the intestinal injury further increases. This suggests that during ECC a process has been initiated that results in ongoing increased intestinal mucosal cell injury, potentially through a prolonged 'functional' hypoperfusion. The mechanisms leading to visceral hypoperfusion during and after ECC in patients undergoing open TAA / TAAA repair remain to be resolved. Such information will be instrumental in developing strategies to prevent the intestinal injury and subsequent systemic inflammatory response induced by ECC with DAP or DAP & SP.

Conclusion

Increased plasma IFABP and LFABP levels, representative of intestinal injury, and elevated plasma IL-6 and IL-8 concentrations, indicative of systemic inflammation, were observed in patients undergoing open TAA / TAAA repair, despite ECC with either

DAP or DAP & SP. Our data show that intestinal injury precedes the development of a systemic inflammatory response and correlates positively with the extent of systemic inflammation, supporting the hypothesis that intestinal tissue injury is involved in the development and extent of a systemic inflammatory response.

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Cardiovascular surgery and organ damage; time to reconsider the role of hemolysis

Iris C Vermeulen Windsant*, Sebastiaan J Hanssen*,
Wim A Buurman, Michael J Jacobs

* These authors contributed equally to this review

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ABSTRACT

Cardiovascular surgery with cardiopulmonary bypass is associated with postoperative organ injury which severely affects patient morbidity and mortality. Multiple cardiopulmonary bypass related mechanisms have been linked to the development of tissue damage including hypoperfusion, ischemia/reperfusion and induction of a pro-inflammatory response. Hemolysis, resulting in increased plasma free hemoglobin concentrations is considered an inevitable, but relatively harmless side-effect of cardiopulmonary bypass. Recently however, evidence is mounting that plasma free hemoglobin scavenges intravascular nitric oxide, thereby attenuating its bioavailability. A significant reduction in nitric oxide, the most important endogenous vasodilator, impairs tissue perfusion and induces organ injury development. Moreover, urinary free hemoglobin contributes to renal damage specifically by catalyzation of reactive oxygen species formation. In this review, the effects of increased free hemoglobin levels on nitric oxide metabolism are discussed. Additionally, we review the role of free hemoglobin in organ injury development, potential sources of free hemoglobin during cardiovascular surgery, as well as therapeutic options to attenuate the consequences of hemolysis. We propose that hemolysis is more than an innocent bystander effect of cardiopulmonary bypass assisted surgery. Therapeutic interventions attenuating the effects of hemolysis seem crucial in the reduction of postoperative morbidity and mortality after cardiovascular surgery.

INTRODUCTION

Cardiovascular surgery with extracorporeal circulation is associated with considerable postoperative morbidity and mortality, especially in patients undergoing complex procedures like combined coronary artery bypass grafting (CABG) and valve surgery, Bentall procedures, and open repair of thoracic and thoracoabdominal aortic aneurysms. These patients are at high risk for developing major complications such as acute kidney injury,¹⁻⁶ pulmonary dysfunction,^{7,8} sepsis, and multiple organ failure.⁹

The pathophysiology underlying these complications has been extensively studied in order to develop specific prevention and treatment strategies. The cardiopulmonary bypass (CPB) circuit has been associated with the development of tissue damage due to insufficient oxygen delivery through hemodilution,¹⁰ ischemia/reperfusion,¹¹ and hypoperfusion.^{12,13} Cardiotomy suction during CPB has been shown to be a source of lipid microemboli forming small vascular occlusions in several tissues, including brain, kidney, spleen and muscle.^{14,15} Furthermore, the non-endothelial surface of the CPB-system initiates a pro-inflammatory response which deteriorates cellular function, for instance the function of renal tubular cells.^{16,17} Indeed, the use of a mini-cardiopulmonary bypass system, reducing the pro-inflammatory contact surface area and hemodilution, attenuates the release of intestinal and renal tissue damage markers as compared to a normal CPB circuit in cardiac surgery patients.¹⁸ Similarly, the incidence of liver injury and kidney injury is significantly reduced in patients undergoing CABG without CPB (off-pump) in comparison with on-pump CABG.^{19,20} Nevertheless, although off-pump surgery has gained popularity world-wide, CPB-assisted surgery is still widely used. To reduce CPB-related morbidity and mortality, successful efforts have been made to increase CPB biocompatibility and flow performance. Unfortunately, this has not led to a significantly decreased incidence of organ dysfunction after cardiovascular surgery.²¹ This underscores the need for further clarification of underlying pathophysiological mechanisms of tissue damage and dysfunction in this setting.²¹

A common consequence of CPB is hemolysis, considered an inevitable, but relatively harmless phenomenon. Hemolysis is principally caused by mechanical shear stress within the perfusion circuit and results in the release of hemoglobin into the circulation.^{22,23} The role of this cell-free plasma hemoglobin (fHb) in the development of organ injury has gained increasing interest since a direct relation between hemolysis, impaired vascular function, decreased organ perfusion, and organ dysfunction has been reproducibly shown in experimental animal models and in chronic hemolytic diseases in men.^{24,25} Most recently, Meyer *et al.* showed that fHb due to hemodialysis-induced hemolysis impaired vascular function in patients, indicating a role for fHb in the development of microcirculatory dysfunction during

acute and transient hemolysis.^{26,27} Moreover, the results of Meyer *et al.* substantiate our recent findings that acute hemolysis during major aortic surgery was independently associated with proximal renal tubular damage and postoperative acute kidney injury.²⁸ Therefore, we propose hemolysis to be an important, but generally unrecognized, contributor to the development of organ injury during surgical procedures associated with hemolysis, such as cardiovascular surgery.

The interfering role of fHb in intravascular nitric oxide (NO) metabolism is believed to play a critical role in the development of microcirculatory impairment, organ damage, and organ dysfunction.²⁴ Therefore, in this review we will discuss a) the effects of fHb on intravascular nitric oxide bioavailability, b) the role of fHb in the induction of microcirculatory dysfunction and organ damage, c) the potential sources of fHb during cardiovascular surgery, and d) therapeutic options to attenuate the consequences of hemolysis during CPB-assisted surgery.

INCREASED PLASMA FREE HEMOGLOBIN DUE TO HEMOLYSIS REDUCES INTRAVASCULAR NITRIC OXIDE BIOAVAILABILITY

Upon intravascular destruction of red blood cells (RBC), fHb enters the circulation. FHb gets bound to either haptoglobin or is oxidized to methemoglobin. The hemoglobin-haptoglobin complex is rapidly cleared from the circulation through endocytosis by the surface scavenger receptor CD163 expressed on monocytes and tissue macrophages. This way, haptoglobin prevents accumulation of plasma fHb under physiological circumstances.²⁹ This effect was illustrated in patients undergoing cardiac surgery in whom intravenous administration of haptoglobin significantly reduced circulating fHb levels.³⁰ Free heme, another by-product of hemolysis, is released during oxidation of free hemoglobin and is scavenged by circulating hemopexin. Subsequently, heme-oxygenase (HO)-1, activated due to reduced microcirculation, degrades heme to carbon monoxide, biliverdin, and iron mainly in the liver and spleen.³¹ This cytoprotective induction of HO-1 by the microvasculature has been shown to modulate inflammation in patients after cardiac surgery with CPB, which may benefit patient recovery postoperatively;³² furthermore, the induction of HO-1 inhibits vascular inflammation and vaso-occlusion in transgenic sickle mice.^{32,33} Since both haptoglobin and hemopexin are not recycled after clearance of hemoglobin-haptoglobin or heme-hemopexin complexes, excessive RBC lysis rapidly exhausts their storage pools. This results in enhanced levels of fHb and free heme, both harmful products. First of all, free heme is able to react with endogenous hydrogen peroxide, thereby forming toxic free radicals which are involved in the induction of pro-oxidant damage.³⁴ Secondly, oxygenated fHb has been shown to be a potent scavenger of NO, the most important endogenous vasodilator. The fast ($6 - 8 \times 10^7$ mol/l/s) and irreversible reaction of

oxygenated fHb with NO results in conversion of fHb to methemoglobin, and conversion of NO to nitrate. Circulating fHb is also present in a deoxygenated form. Such deoxygenated fHb also scavenges NO, forming nitrosyl-Hb, but this reaction is slower (10^7 mol/l/s) and reversible.^{24,29} As a result, hemolysis significantly impairs NO-bioavailability, potentially inducing microcirculatory dysfunction.^{35,36} *In vivo*, fHb associated NO-scavenging was correlated to systemic vasoconstriction and a reduction in renal function in a canine hemolysis model.²⁵ In patients with chronic high fHb levels due to sickle cell disease, forearm blood flow responses were reduced by 80% after infusion of the nitric oxide donor sodium nitroprusside, compared to patients with below average fHb levels.²⁴ Thirdly, hemolysis also results in release of arginase-1, an enzyme that converts L-arginine, the substrate for NO synthesis, to ornithine.³⁷ This way, hemolysis causes not only scavenging of NO, but also, theoretically, prevents new NO formation. In practice, however, we showed that arginase-1 release during surgery with CPB does not affect the arginine-ornithine ratio (unpublished data). This implies that arginase levels during this type of surgery are not high enough to affect arginine levels and attenuate NO-synthesis.³⁸

CELL FREE HEMOGLOBIN CONTRIBUTES TO MICROCIRCULATORY DYSFUNCTION THROUGH NO-SCAVENGING, POTENTIALLY INDUCING HYPOXIC TISSUE DAMAGE

The role of hemolysis in organ damage development was already described in the mid nineteen seventies.^{24,39} These studies focused on acute kidney injury, since glomerularly filtered urinary fHb, instead of plasma fHb, was considered the culprit of organ injury induction. Therefore, at that time, only the kidney was believed to be at risk for fHb induced damage. Two mechanisms were proposed to underlie the association between hemolysis and renal tissue damage development. First, urine fHb-derived free iron and heme catalyze the generation of reactive oxygen species which damage the renal tubular epithelium.⁴⁰ Indeed, administration of the iron scavenger deferoxamine attenuated glomerular and tubular dysfunction induced by intravenous fHb administration in rats.⁴¹ Similarly, a reduced intravascular iron scavenging capacity – reflected by low plasma ferritin concentrations – was associated with acute kidney injury after human cardiovascular surgery.⁴² Second, intra-tubular fHb precipitation and heme cast formation in the acidic ultrafiltrate was considered to obstruct the tubular lumen, reducing glomerular filtration.^{40,43} Subsequent prevention of cast formation by urine alkalinization reduced tubular injury and glomerular dysfunction after intravenous fHb infusion in rats.⁴⁰

The discovery of NO-scavenging property of circulating plasma fHb by the group of Gladwin provided a complementary explanation for hemolysis-induced organ injury.²⁴

For the first time, circulating fHb was recognized as a key player in the pathophysiology of complications in patients with chronic hemolytic disorders, such as sickle cell disease and malaria infection.²⁴ Furthermore, the reported adverse effects associated with administration of hemoglobin based oxygen carriers – which basically consist of fHb – could be explained by intravascular NO-scavenging through fHb.⁴⁴ The negative effects of increased plasma fHb have been confirmed by many studies since 2002, in both animals and patients. For example, hemolysis induced by water infusion or direct intravascular fHb administration in dogs was associated with a significant increase in plasma NO consumption and simultaneous enhanced systemic vascular resistance. These effects were attenuated by NO-inhalation (which converts plasma fHb into the less bioactive molecule methemoglobin in the pulmonary circulation), supporting a causal role for NO-scavenging by fHb.²⁵ In humans, fore-arm blood flow responses to intra-arterial infusion of sodium nitroprusside, a NO-donor, were negatively correlated to plasma fHb levels in sickle cell patients.^{24,25}

The potential role of plasma fHb in organ injury development is further supported by our observation that plasma fHb levels are significantly associated with renal proximal tubular damage during CPB-assisted major aortic surgery.²⁶ Moreover peak plasma fHb levels significantly predicted postoperative acute kidney injury. We could not detect fHb in urine samples during the perioperative period, indicating that urine fHb is not a major contributor to renal tubular injury development in this setting.²⁶ Also, fore-arm blood flow responses after infusion of sodium nitroprusside at the time of peak plasma fHb concentrations were significantly reduced, as compared to the response measured when fHb-levels were normalized. This observation further underscores a potential causal role of fHb-induced tissue perfusion impairment during surgery (unpublished data). In addition, we have shown that fHb induces intestinal microcirculatory dysfunction and tissue integrity loss in a rat hemolysis model.⁴⁵

SOURCES OF CIRCULATING FREE HEMOGLOBIN DURING CARDIOVASCULAR SURGERY WITH CARDIOPULMONARY BYPASS

Hemolysis can principally be attributed to three sources during cardiovascular surgery; the CPB, the cell saver system, and (massive) red blood cell transfusion.

Cardiopulmonary bypass

Cardiopulmonary bypass inflicts (sub)lethal RBC damage through turbulence and shear stress within the pump, tubes, connectors, cannula, reservoirs, and the oxygenator.²³ Blood-air contact, blood-nonendothelial surface contact, wall impact forces, use of positive and negative pressures to assist venous drainage, and the use of an integrated cardiotomy suction reservoir all contribute to intraoperative hemolysis.^{23,46,47} Next to

CPB-composition, CPB-duration is considered to influence the degree of hemolysis, with longer CPB-times resulting in increased RBC-lysis.⁴⁸ In support of this phenomenon, we found a positive and statistically significant correlation between total fHb release in the perioperative period and CPB-time in a group of 54 patients undergoing CPB-assisted major aortic surgery (**Figure 4.1**). Next to direct red blood cell lysis, the CPB-system induces sub-lethal RBC-injury.^{49,50} Such sub-lethally damaged RBCs are more prone to lysis *in vivo*.^{23,51} Delayed lysis of sub-lethally damaged RBCs could explain the continuing increase of plasma fHb after cessation of CPB (**Figure 4.2**).

Figure 4.1 Association between cardiopulmonary bypass time and extent of hemolysis. A correlation-analysis between cardiopulmonary bypass time and total free hemoglobin release (defined as the area under the curve) was performed in fifty-four patients (N = 54) undergoing open surgical repair of thoracoabdominal aortic aneurysms with cardiopulmonary bypass. A significant correlation (Pearson $r = 0.33$, $p < 0.05$) was found between cardiopulmonary bypass time and plasma free hemoglobin release. Abbreviations: CPB, cardiopulmonary bypass; fHb, free haemoglobin.

Figure 4.2 Hemolysis during open surgical repair of thoracoabdominal aortic aneurysms with cardiopulmonary bypass. Plasma free hemoglobin levels increased during surgery and continued to increase significantly in the early postoperative period. This indicates ongoing lysis of red blood cells after cardiopulmonary bypass has stopped. * / *** $p < 0.05$ / 0.001 compared to preoperative levels. # / ## $p < 0.05$ / 0.01 compared to end-CPB-levels. Abbreviations: CPB, cardiopulmonary bypass; fHb, free hemoglobin; R, reperfusion

Cell saver

Cell salvage devices are additional sources of fHb.^{52,53} Washing and transfusion of damaged autologous shed blood could contribute to increased plasma fHb levels. Although modern cell saver systems are able to remove the major part of fHb during washing, they do not select between intact and damaged RBCs which are prone to lysis in vivo.⁵⁴ This way, autologous blood transfusion could contribute to increased plasma fHb levels during surgery.

Red blood cell transfusion

A last potential source of fHb are stored RBC-concentrates. Storage of erythrocytes results in irreversible morphological changes like reduced membrane deformability and oxygen binding and delivery capacity, in addition to increased adhesiveness, aggregability, and accumulation of pro-inflammatory substances.⁵⁵ These changes are considered to underlie the relation between RBC transfusion and adverse outcome, a phenomenon causing debate about the pros and cons of allogenic RBC administration. Additionally, storage duration of blood products appears to be a critical factor of transfusion-related morbidity and mortality. Transfusion of “old” blood (stored > 14 days) was associated with a significantly higher mortality rate in patients undergoing cardiac surgery, compared to patients receiving RBCs stored for 14 days or less. Transfusion of “older” blood was also significantly related to prolonged ventilatory support, increased incidence of renal failure, septicemia or sepsis, and multiple organ failure.⁵⁵ In line, RBC transfusion was the most reliable predictor of adverse outcome in 11,963 patients undergoing isolated CABG, with postoperative morbidity and mortality being dose-dependently related to RBC transfusion.⁵⁶ We propose that high fHb concentrations due to RBC lysis in stored blood contribute to post-transfusion morbidity and mortality by inducing microcirculatory dysfunction through NO-scavenging. In addition, the shear stress imposed on less viable RBCs within the transfusate, might cause an additional increase of fHb during and after infusion. To study the degree of direct hemolysis of packed RBCs, we measured fHb levels by derivatized spectrophotometry⁵⁷ in samples from sixty randomly collected and transfused packed RBC units (330 ml/U). fHb in the packed RBC supernatant levels averaged $36 \pm 2 \mu\text{M}$ (mean $\pm$ SEM), indicating severe hemolysis. Moreover, storage time and fHb levels were significantly correlated (**Figure 4.3**).

This means that transfusion of especially aged packed RBCs can result in an additional increase in circulating fHb in patients undergoing cardiovascular surgery with CPB. Recently, it was reported that packed RBC transfusion indeed contributed to systemic fHb levels with $7.5 \mu\text{M}$ increase per transfused unit.⁵⁸ Importantly, the supernatant of the packed RBC, which contains the fHb molecules was indeed able to consume NO,

with a strong correlation between fHb levels and NO-consumption.⁵⁸ The contributing effect of RBC transfusion to circulating plasma fHb concentrations may thus be considerable. The median transfusion requirement in a large cohort of cardiac surgery patients was found to be 2 packed RBC units,⁵⁵ and 10 units for patients undergoing open repair for thoracoabdominal aortic aneurysms, respectively.²⁸ Transfusion of 2 or 10 packed cells could increase plasma fHb levels with 15 μM and 75 μM , respectively. These levels are in sharp contrast to the plasma fHb levels of 0.1–0.2 μM in normal healthy volunteers.^{24,58} Next to direct fHb administration via packed RBC transfusion, systemic fHb concentrations could additionally increase due to delayed lysis of sub-lethally damaged RBCs. Lastly, another consequence of RBC storage is that so-called S-nitrosylated hemoglobin (SNO-Hb; NO bound to intracellular hemoglobin) is rapidly depleted. RBCs containing SNO-Hb are believed to contribute to vasodilation under hypoxic conditions through release of NO. Indeed, the capacity of RBCs to induce vasodilation significantly diminished parallel to SNO-Hb depletion. Reconstitution of SNO-Hb by exposure to aqueous NO restored the vasodilatory capacity of transfused RBCs, and improved cardiac blood flow in dogs.⁵⁹

Figure 4.3 Association between storage time and free hemoglobin levels in packed red blood cell transfusion bags. A significant correlation (Pearson $r = 0.43$, $p < 0.001$) was found between packed red blood cell storage time and free hemoglobin concentration in packed red blood cell transfusion bags ($N = 60$) used for transfusion during cardiovascular surgery. These data indicate that prolonged storage time results in more red blood cell injury with concomitant more free hemoglobin being transfused into the patient. Abbreviations: fHb, free hemoglobin

THERAPEUTIC OPTIONS TO ATTENUATE THE ADVERSE EFFECTS OF FHB-MEDIATED NO-SCAVENGING

The discovery of the NO-scavenging capacity of circulating fHb opened a new field of study on therapeutic options in diseases characterized by chronic hemolysis, such as sickle cell disease and malaria.⁶⁰ In this paragraph, we present therapies that we consider to be of value for patients suffering from acute hemolytic episodes as occur during cardiovascular surgery (**Table 4.1**).

Table 4.1 Potential therapeutic options to reduce hemolysis-associated morbidity in patients undergoing cardiovascular surgery

Therapy	Main therapeutic mechanisms	Feasibility	References
NO-inhalation	Oxidates and inactivates fHb in the pulmonary circulation reducing NO-scavenging. Stimulates intrapulmonary formation of NO-donors; enhancing NO bio-availability.	NO-inhalation already applied in sickle cell patients and patients who underwent cardiac surgery. Exact dose and duration of inhalation needs to be studied.	Reiter <i>et al.</i> ²⁴ , Minneci <i>et al.</i> ²⁵ , Kato <i>et al.</i> ⁶⁰ , Cannon <i>et al.</i> ⁶⁷
Nitrite supplementation (oral, intravenously, by inhalation)	NO-donor during reduction, especially during hypoxia and low pH. Mediates cytoprotection through hypoxic vasodilation and decreased formation of reactive oxygen species. Oxidizes fHb, hereby reducing NO-scavenging.	Nitrite successfully used in experimental setting with sickle cell patients. Exact dose, duration, and way of administration needs to be assessed.	Lundberg <i>et al.</i> ⁶² , Shiva <i>et al.</i> ⁶⁹ , Piknova <i>et al.</i> ⁷¹ , Minneci <i>et al.</i> ⁷⁶
Haptoglobin administration	Natural fHb scavenger. Accelerates fHb uptake by monocytes/macrophages and hepatic degradation. Limits renal filtration of fHb.	Costs currently limit clinical applicability. Moreover, haptoglobin potentially does not limit NO-scavenging by fHb.	Tanaka <i>et al.</i> ³⁰ , Lim <i>et al.</i> ⁸¹ , Azarov <i>et al.</i> ⁸² , Boretti <i>et al.</i> ⁸³
Arginine and citrulline supplementation	Enhances substrate delivery for NO-formation through the nitric oxide synthases.	Arginine used experimentally in 10 patients with sickle cell disease. Dose and duration of arginine and citrulline administration unknown.	Morris <i>et al.</i> ⁸⁶ , Luiking <i>et al.</i> ⁸⁸
Carbon monoxide inhalation	Regulates vascular tone and induces vasodilation at low doses. Reduces the pro-inflammatory response. Relatively inert and does not form reactive oxygen species.	No human data on applicability in the setting of cardiovascular surgery.	Belcher <i>et al.</i> ³³
Endothelin receptor blockade	Limits endothelin-1 induced vasoconstriction.	Only tested in a mouse sickle cell model.	Sabaa <i>et al.</i> ⁹⁰

The direct (intravascular) therapeutic use of NO itself is greatly impeded by its extremely short half life of 0.05 - 1.8 msec. *in vivo*.⁶¹ Therefore, either inactivation of fHb, or enhancement of the NO(-donor) pool, have potential in reducing the incidence and severity of complications of hemolytic diseases. We mainly focus on three potential therapeutic interventions (**Figure 4.4**); NO-inhalation, nitrite supplementation, and haptoglobin administration as we consider these therapeutic options to be most clinically relevant at this point of time. In addition, we will discuss

three more experimental therapies: arginine and citrulline supplementation, CO-inhalation, and endothelin receptor blockade. For a more complete discussion of therapeutic options in chronic hemolytic disease we would like to refer to Kato *et al.*⁶⁰ and Lundberg *et al.*⁶²

Figure 4.4 Potential sources and effects of plasma free hemoglobin during cardiovascular surgery with cardiopulmonary bypass, and therapeutic options to attenuate hemolysis-induced organ damage. Intravascular hemolysis during cardiovascular surgery can be attributed to the cardiopulmonary bypass, transfusion of red blood cells, and cell-saver use (left). Lysis of red blood cells results in increased circulating plasma free hemoglobin (fHb) levels (orange squares). Under physiological circumstances, fHb is rapidly cleared by the fHb scavenger haptoglobin. Haptoglobin-hemoglobin complexes bind to CD-163 expressed by monocytes and macrophages, initiating endocytosis and degradation (middle, bottom). Haptoglobin is not recycled so excessive hemolysis depletes haptoglobin storages rapidly. Non-scavenged fHb potently binds circulating nitric oxide (NO), thereby limiting its bioavailability. This way, high fHb levels increase the NO-scavenging capacity of blood, causing impaired vasodilation due to vascular NO-shortage. Decreased vasodilation contributes to impaired tissue perfusion and development of organ damage and organ dysfunction (right, bottom). The adverse effects of fHb may be counteracted by either increasing haptoglobin levels to support fHb scavenging, or enhancing the NO(-donor) pool to increase NO-bioavailability. Haptoglobin can be administered intravenously or haptoglobin synthesis may be up-regulated through corticosteroid administration (center, bottom). The NO(-donor) pool could be increased by oral or intravenous nitrite administration which is oxidized to NO under low oxygen tension and/or low pH. NO-inhalation inactivates fHb in the pulmonary circulation (or oxygenator in the cardiopulmonary bypass circuit) by transforming fHb to bioinactive methemoglobin (center, top). This way, scavenging and inactivation of fHb, and supplementation of NO prevents the adverse effects of plasma fHb during cardiovascular surgery (right, top). Abbreviations: Hp-Hb-complex, haptoglobin-hemoglobin complex; fHb, free hemoglobin; NO, nitric oxide; methHb, methemoglobin. See separate inlay for the colour figure.

NO-inhalation

Intravascular conversion of plasma fHb into a less bioactive molecule is an interesting option to reduce the adverse consequences of increased fHb concentrations. NO gas inhalation results in pulmonary oxidation of fHb into methemoglobin, which does not scavenge NO, reducing NO consumption in plasma. In a canine hemolysis model, NO-inhalation attenuated the pulmonary and systemic vasoconstrictor effects of fHb.²⁵ In patients with sickle cell disease, NO-inhalation of 80 ppm for 1.5 hours reduced pain during vaso-occlusive crisis, diminished plasma NO-consumption and increased methemoglobin levels, indicating oxidation of fHb.²⁴ Moreover, inhalation of 80 ppm NO for 4 hours in children with sickle cell disease was not associated with any toxic side-effects such as hypotension, clinically significant decreases in SpO₂, significant increases in methemoglobin, or toxic concentrations of NO₂.⁶³ Even continuous NO-inhalation at 40 ppm for 3.2 days in a patient with sickle cell disease with multi-organ involvement was not associated with adverse side-effects and markedly improved her clinical state.⁶⁴ Lastly, mixing of up to 20 ppm NO gas with normal ventilation gas showed therapeutic potential in adult patients with cardiac surgery associated pulmonary hypertension by reducing right ventricular afterload and prevention of right ventricular failure.^{65,66} Next to fHb oxidation, NO-gas inhalation also results in the formation of relatively stable NO-species in the lung. Longer intravascular half-times of these NO-carriers, such as nitrite, enable transport of NO in the blood, mediating extra pulmonary effects of NO-gas inhalation.^{67,68} In conclusion, we consider NO-inhalation a promising and potentially easy applicable, therapeutic option to attenuate the adverse effects of fHb-mediated NO-scavenging during cardiovascular surgery. Ideally, the dose of NO-inhalation could even be adjusted based on intraoperative fHb measurements, which are already routinely performed at our institution.

Nitrite supplementation

Another promising candidate for therapeutic use is the anion nitrite (NO₂⁻), long believed to be merely an inert oxidation product of NO. Recent studies, however, provided evidence for the existence of multiple nitrite reducing pathways in which nitrite is converted back to NO, making nitrite an important NO-donor.^{62,69,70} Furthermore, nitrite is able to oxidize fHb in plasma, potentially limiting its capacity to scavenge NO.⁷¹ The enzymatic and non-enzymatic pathways of nitrite reduction include (free) deoxyhemoglobin and deoxymyoglobin, xanthine oxidoreductase, protons, ascorbate, and polyphenols.⁶² The process of NO-reduction is most efficient at low oxygen tension and low pH and thus occurs, preferably, during hypoxia and/or anoxia.^{70,72,73} This is of particular importance since NOS-activity is greatly limited at low-oxygen tensions.⁷⁴ In adult patients suffering from sickle cell anemia, infusion of

0.4, 4, and 40 μM nitrite into the brachial artery led to a dose-dependent increase in fore-arm blood flow up to 77%.⁷⁵ In a canine hemolysis model, sodium nitrite increased blood flow in a similar way.⁷⁶ Next to promoting vasodilation, nitrite has been shown to exert potent cytoprotection in the liver, heart, and brain in several animal models of ischemia-reperfusion.⁷⁷ The way by which nitrite mediates cytoprotection in this setting is as yet unresolved but believed to be dual. First, nitrite enables hypoxic vasodilation as stated above. Second, nitrite is able to nitrosate complex I of the mitochondrial electron transport chain, inhibiting its activity and decreasing the formation of reactive oxygen species in the reperfusion phase.⁶⁹ Considering these properties of nitrite, it has been suggested to have therapeutic value in various diseases, such as sickle cell disease, stroke, myocardial infarction, and organ transplantation. Nevertheless, the optimal nitrite dose for human systemic administration has not yet been clarified. Interestingly, the effect of nitrite may be greater at low plasma concentrations ($< 200 \text{ nM}$) and is lost at high plasma levels exceeding $1000 \mu\text{M}$.^{78,79} Furthermore, it was reported that the reaction between oxyfHb (in contrast to deoxyfHb) and nitrite could initiate an autocatalytic free radical chain leading to unwanted oxidative damage.⁸⁰ Piknova *et al.* addressed this issue and concluded that free radical formation in plasma during the reaction of pharmacological doses of nitrite (up to $120 \mu\text{M}$) with clinically relevant levels of fHb ($30 \mu\text{M}$), would be highly unlikely.⁷¹ In conclusion, we consider nitrite to have potential in patients undergoing cardiovascular surgery to prevent and treat hemolysis-associated morbidity. Nevertheless, the optimal dose and application must be further studied.

Haptoglobin administration

Administration of haptoglobin, being the physiological fHb scavenger, appears to be a logical choice in reducing fHb concentrations. Haptoglobin targets fHb for degradation in the liver, monocytes and macrophages. Furthermore, haptoglobin prevents glomerular filtration of fHb, reducing fHb-induced kidney damage. Increased fHb levels during cardiac surgery have been associated with total depletion of haptoglobin,³⁰ enhancing the NO-scavenging capacity of plasma. Indeed, haptoglobin knock-out mice were more sensitive to the adverse effects of phenylhydrazine-induced hemolysis.⁸¹ Renal DNA damage was significantly higher and glomerular filtration function (reflected by poorer renal clearance of ^3H -inulin) was significantly lower in haptoglobin knock-out mice compared to haptoglobin^{+/+} mice. Interestingly, administration of vasodilators restored glomerular filtration, implicating renal vasoconstriction to be the major contributor to hemolysis-induced acute kidney injury. This supports NO-scavenging by plasma fHb. In addition, haptoglobin administration in patients undergoing cardiac surgery with plasma fHb levels surpassing $2.3 \mu\text{M}$, was associated with a reduction of renal tubular damage.³⁰ Nevertheless, this positive effect of

haptoglobin was attributed to a decrease in urine fHb levels and a subsequent attenuation of oxidative renal damage.

Haptoglobin administration is an interesting therapeutic option, but its clinical application might be limited. First, it has been recently found that, *in vitro*, the hemoglobin – haptoglobin complex still potently scavenges NO at the same rate as fHb. Although binding of fHb to haptoglobin increases the rate of uptake by monocytes and macrophages by 2-fold (hereby disabling NO-scavenging), it is questionable whether this increased uptake would affect NO-bioavailability.⁸² Second, the costs involved in retrieving or producing the amounts of haptoglobin necessary for clinical application currently limits wide-spread implementation. Recently, Boretti and *et al.* made use of the fact that the haptoglobin promoter gene contains glucocorticoid-responsive elements. Administration of 4 mg/kg prednisone twice daily for three days increased plasma haptoglobin levels 6-fold in dogs.⁸³ Importantly, the glucocorticoid stimulation of haptoglobin synthesis prevented the fHb-induced increase in mean arterial pressure in dogs after fHb infusion, most probably due to scavenging of fHb by haptoglobin.⁸³ This sheds new light on the longstanding debate whether corticosteroid administration during cardiac and cardiovascular surgery is useful.^{84,85} In summary, haptoglobin could be useful to reduce kidney damage due to intratubular fHb toxicity. The effect on NO-scavenging may, however, be limited.

OTHER POTENTIAL THERAPIES

We consider NO-inhalation and nitrite supplementation to be promising therapeutic interventions since they can be used successfully in the acute setting of cardiovascular surgery. Notwithstanding, several other therapeutic modalities may be of value in patients at risk for acute hemolytic episodes.

Arginine and citrulline supplementation

L-arginine is the natural nitrogen donor for NO-synthesis and arginine supplementation enhances NO-formation. Arginine therapy at a dose of 0.1 g/kg three times a day for 5 days in 10 adult patients with sickle cell anemia and pulmonary hypertension resulted in a significant decrease (15.2%) of pulmonary arterial systolic pressures implicating vasodilation.^{86,87}

Another interesting approach would be administration of citrulline, a substrate for de novo arginine synthesis. It has been suggested that in case of high arginase-1 levels, as occur during hemolysis, citrulline supplementation might restore the intracellular arginine balance and promote nitric oxide production.⁸⁸

However, it remains unknown whether, and to what extent, arginine or citrulline supplementation is beneficial in patients with acute hemolysis during cardiovascular surgery.

Carbon monoxide inhalation

Carbon monoxide (CO) is produced during breakdown of the heme-ring of fHb which is mediated by HO-1. Inhaled CO, at low doses up to 250 ppm, has been shown to reduce vaso-occlusion in a mouse sickle cell model. CO even mimics some of the functions of NO like inhibition of platelet aggregation and activation, regulation of vascular tone, and reduction of a pro-inflammatory response.³³ CO is relatively inert in contrast to NO, which is able to react with intravascular reactive oxygen species forming the highly reactive peroxynitrite (ONOO⁻).⁸⁹ Therefore, CO has been proposed to be even more effective in hemolytic disease than NO, but this has not been substantiated by human studies.

Endothelin receptor blockade

Endothelin-1 is a very potent vasoconstrictor and its secretion is repressed by NO. A diminished NO-bioavailability due to hemolysis counterbalances this negative feedback, resulting in enhanced endothelin-1 levels and vasoconstriction. A mouse sickle cell model provided evidence for beneficial effects of endothelin receptor blockade on renal blood flow, inflammation, and vascular congestion in the lungs and kidneys.⁹⁰ Reduction of inflammation would be an additional positive effect in patients subjected to cardiopulmonary bypass, which induces a pro-inflammatory response. Nevertheless, further research is essential to find out whether short term endothelin receptor blockage is beneficial in this setting, since it is very different compared to sickle cell anemia.

In conclusion, there are several promising therapeutic interventions to attenuate the adverse effects of increased plasma fHb levels in patients suffering from acute hemolytic disease, including patients undergoing surgery with cardiopulmonary bypass, trauma patients, and patients necessitating long-term extracorporeal support for instance extracorporeal membranous oxygenation (ECMO) or hemodialysis.^{91,92}

HEMOLYSIS-INDUCED ORGAN INJURY IN CARDIOVASCULAR SURGERY; SUMMARY AND FUTURE PERSPECTIVES

As evaluated in this review, hemolysis during cardiopulmonary bypass harbors a latent adverse effect. The NO-scavenging effect of plasma fHb contributes to deleterious effects of CPB, such as hypoperfusion and ischemia/reperfusion, thereby further hampering tissue perfusion and resulting in organ injury and dysfunction. These

findings shed new light on the pathophysiology and preventive measures of organ injury during on-pump cardiovascular surgery (Figure 4.4).

CONCLUSION

Evidence is mounting that enhanced circulating levels of plasma fHb are detrimental, not only in chronic hemolytic diseases, but also in the acute setting of cardiovascular surgery. FHb-induced perturbations in microcirculatory blood flow and subsequent hypoperfusion or even ischemic damage, complemented by urine fHb-induced oxidative stress to renal tissue cells, should be acknowledged as an important risk factor for organ injury development in patients undergoing cardiovascular surgery. Patients undergoing such procedures are at increased risk for developing post-operative organ injury with worse patient outcome. Circulating fHb appears to be an important determinant in organ injury development which offers a new therapeutic opportunity to reduce post-operative morbidity and mortality in patients. Interventional studies using NO-inhalation, nitrite administration or haptoglobin suppletion should be performed to establish the causal role between plasma fHb, NO-bioavailability, and organ injury in this particular setting.^{25,30,93,94} Furthermore, such interventional studies will provide valuable information for improvement of patient outcome. Lastly, the role of fHb in organ injury development is not only of importance for patients undergoing cardiovascular surgery but also for other patient groups at risk for hemolysis such as trauma patients, hemodialysis patients, and patients requiring long-term extracorporeal oxygenation or extracorporeal life-support.

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Hemolysis compromises nitric oxide-dependent
vasodilatory responses in patients
undergoing major cardiovascular surgery

Sebastiaan J Hanssen, Marcel C van de Poll, Alfons J Houben,
Iris C Vermeulen Windsant, Maarten G Snoeijs, Otto Bekers,
Wim A Buurman, Michael J Jacobs

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ABSTRACT

Background

The hemolytic products cell-free oxyhemoglobin (fHb) and arginase-1 reduce nitric oxide (NO) bioavailability by scavenging of NO and by degrading the NO precursor arginine to ornithine, respectively. In this study we evaluated the relevance of hemolysis to NO-dependent blood flow in patients undergoing cardiovascular surgery.

Methods

Plasma fHb, arginase-1 and amino acid concentrations were measured perioperatively. Forearm blood flow (FBF) responses to the intra-arterial administered NO-donor sodium nitroprusside (SNP) and the endothelium-dependent vasodilator acetylcholine (ACh) were measured by venous occlusion plethysmography.

Results

When peak values plasma fHb and arginase-1 were found, vascular dilatation to SNP, but not ACh, was significantly reduced compared to 1 day postoperatively, when fHb had returned to baseline levels ($p < 0.05$). Interestingly, plasma fHb concentration was inversely correlated to FBF responses to SNP ($R_s = -0.93$, $p < 0.001$). In contrast, the increase in arginase-1 was not biologically relevant since the ratio of plasma arginine to ornithine remained constant.

Conclusion

We conclude that hemolysis with concomitant release of fHb during cardiovascular surgery is associated with impaired NO-dependent forearm blood flow.

INTRODUCTION

Intravascular hemolysis is associated with disturbances in endothelial nitric oxide (NO) metabolism, the key system in regulating vascular tone and microcirculatory blood flow.¹ First, oxyhemoglobin (fHb) released from injured erythrocytes reduces NO bioavailability by scavenging NO. Oxyhemoglobin in its ferrous form binds NO to generate ferric hemoglobin, also known as methemoglobin, and bioinactive nitrate ($\text{HbFe}^{2+}\text{-O}_2 + \text{NO} \rightarrow \text{HbFe}^{3+} + \text{NO}_3^-$). Second, arginase-1 release from hemolytic erythrocytes has been suggested to decrease NO production by depletion of plasma arginine, the precursor for NO synthesis, with a concomitant increase of plasma ornithine, the product of arginase-1 activity. Both fHb and arginase-1 may impair NO bioavailability resulting in a compromised microcirculation. Evidence is mounting that hemolysis is an important factor in the pathogenesis of compromised microcirculation in chronic hereditary or acquired hemolytic diseases such as sickle cell disease and falciparum malaria infection.¹

Hemolysis is unwanted but not uncommon in cardiovascular surgery. Extracorporeal circulation (ECC) is an inevitable part of open cardiac and thoracic- or thoracoabdominal aortic surgery and known to result in hemolysis.² This type of surgery is also associated with considerable blood loss, leading to massive transfusion requirements. Hemolysis during storage of red blood cells intended for transfusion is associated with increased mortality rate in patients undergoing massive allogeneic transfusion. Furthermore, storage results in lower viability of erythrocytes intended for transfusion, which has been associated with clinical adverse events in the recipient such as postoperative infection, pulmonary and renal complications and multiple organ failure.³ Allogeneic transfusion requirements may be reduced by the application of cell-salvage devices during cardiovascular surgery. However, such devices lead to injury of red blood cells intended for autologous transfusion.⁴

Aim of the present study was first to investigate the extent of hemolysis and the concomitant release of fHb and arginase-1 in patients undergoing cardiovascular surgery with extracorporeal circulation and massive transfusion including cell-salvage. Next, forearm vascular smooth muscle responses to exogenous NO in the presence of plasma fHb were evaluated. Lastly, the effect of elevated plasma arginase-1 on plasma arginine and ornithine levels was evaluated.

METHODS

Study subjects

The local institutional review board approved the study and written informed consent was obtained from every patient (N = 19) prior to surgery. Patients, 18 years or older,

undergoing open thoracic aortic aneurysm (TAA) or thoracoabdominal aortic aneurysm (TAAA) repair at the Maastricht University Medical Centre (Maastricht, the Netherlands) were continuously included in the study. Exclusion criteria were pregnancy, morbid obesity (> 120 kg or BMI > 35 kg/m²), prolonged or high dose corticosteroid use, insulin-dependent diabetes mellitus, metastases, hematological malignancies or chemotherapy, pre-existent renal failure, liver cirrhosis, pre-existent urea cycle disorders, or any other condition that, in the physician's judgment, would make the patient an unsuitable candidate for participation in this study. In the first consecutive series of 10 patients plasma fHb, arginase-1 and amino acid levels were evaluated; then a consecutive series of 9 patients was included in the study to conduct forearm blood flow measurements as described below. This study design was chosen to eliminate possible confounding factors resulting from interference of the NO-donor sodium nitroprusside used in the forearm blood flow study on measurement of fHb levels and potentially NO metabolism, possibly affecting amino acid levels. In all cases ECC was applied using a centrifugal pump with oxygenation (Jostra Rotaflow with Jostra Quadrox, Maquet Cardiopulmonary AG, Hirrlingen, Germany) in combination with a cell-saver (CATS, Fresenius SE, Bad Homburg, Germany). Surgical procedures are extensively described elsewhere.^{5,6} Intraoperatively and post-operatively the motor evoked potentials were constantly monitored in our patients to evaluate spinal cord function. During ICU-stay, using inotropic drugs when necessary, the mean arterial pressure was kept between 70 and 90 mmHg, in general this was sufficient to reach adequate motor evoked potentials.

All patients had an indwelling radial artery catheter that was placed as part of standard anesthetic care. Postoperatively, all patients were transported to the Cardiothoracic Intensive Care Unit for postoperative monitoring and circulatory and ventilatory support. Patients were warmed up to 37°C before leaving the OR, and were kept at 37°C during their ICU stay, using filtered-air warming blankets when necessary. Patient characteristics and intraoperative data are reported in **Table 5.1**.

Blood sampling, sample processing and laboratory analysis

Arterial blood was drawn preoperatively and at several time points during and after surgery and collected in vacuum tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ). Whole blood was centrifuged (3500 x G, 4°C, 15 min), 100µl plasma was deproteinized with 8 mg sulphosalicylic acid (SSA) and stored at -80°C, the remainder was stored untreated (-80°C) for fHb and arginase-1 analysis. fHb concentrations were measured by derivative spectrometry as described elsewhere.⁷ Arginase-1 concentrations were measured using Enzyme-Linked Immuno Sorbent Assay (ELISA)⁸ kindly provided by Hycult biotechnology, Uden, the Netherlands. The detection limits of the assays were 2 µmol/l (fHb) and 21 µg/l (arginase-1). Amino acid levels were

analyzed in SSA samples by high performance liquid chromatography as described before.⁹

Table 5.1 Characteristics of patients undergoing open TAA or TAAA repair

	Initial study (N = 10)	FBF study N = 9)
Patients (N)	10	9
TAA	0	2
TAAA*		
TAAA I	0	1
TAAA II	3	2
TAAA III	4	2
TAAA IV	3	2
Male (N)	7	5
Age (Years)	68.0 ± 2.2	63.7 ± 2.0
(Ex-) Smoker with >10 pack-years (N)	8	6
Hypertension (N)	8	7
Dyslipidaemia (N)	6	4
NIDDM (N)	1	-
ACC time (min)	89.7 ± 14.3	100.6 ± 28.4
ECC time (min)	161.8 ± 15.5	139.9 ± 35.5
Flow ECC (L/min)	2.5 ± 0.1	2.8 ± 0.6
Patient hematocrit during ECC (L/L)	0.27 ± 0.01	0.24 ± 0.01
Estimated blood loss (L)	16.6 ± 2.6	14.3 ± 5.1
Infused fresh frozen plasma (L)	2.9 ± 0.5	1.7 ± 0.7
Infused crystalloids (L)	6.5 ± 0.9	5.4 ± 0.9
Infused colloids (L)	5.1 ± 1.0	3.4 ± 0.7
Transfused allogeneic blood (L)	3.3 ± 0.5	3.3 ± 1.0
Transfused autologous cell-salvage blood (L)	5.9 ± 1.1	5.0 ± 1.8
Total operation time (min)	424.2 ± 25.5	400.4 ± 44.8

Abbreviations: TAA, thoracic aortic aneurysm; TAAA, thoracoabdominal aortic aneurysm; NIDDM, non-insulin dependent diabetes mellitus; ACC, aorta cross clamp; ECC, extracorporeal circulation. *TAAA classification is according to Crawford. Continuous variables are expressed as mean ± SEM.

Forearm blood flow measurements and NO reactivity

Forearm blood flow (FBF) served as a model for organ perfusion and was measured using venous occlusion plethysmography as described previously.¹⁰ FBF measurements in the non-dominant arm were conducted immediately upon arrival at the Cardiothoracic Intensive Care Unit approximately 2 hours following cessation of ECC and massive transfusion including cell-salvage, and again 24 hours later.

FBF was measured before and during infusion of vasoactive substances in the brachial artery of the study arm. An endothelium-independent vasodilator (NO-donor sodium nitroprusside (SNP), Spruyt Hillen BV, IJsselstein, the Netherlands), as well as an endothelium-dependent vasodilator (Acetylcholine (ACh), Clinalfa, Läufelfingen, Switzerland) was used. Both substances were infused in two accumulating dosages

(SNP 1 µg/min and 10 µg/min; ACh 3 µg/min and 30 µg/min; 3 min/dosage) in all patients, during which the mean arterial pressure at that time remained between 70 - 90 mmHg. The order of SNP and ACh was randomly chosen with a washout period of 30 minutes between SNP and ACh infusion.

Statistics

Changes versus preoperative values in plasma fHb, arginase-1 and amino acid concentrations were analyzed using repeated-measures ANOVA with post hoc Dunnet's multiple comparison test using pre-operative values as control. Changes in FBF measurements were tested using Wilcoxon signed rank tests. Statistical calculations were made using Prism 4.0 for Windows (GraphPad Software Inc. San Diego, CA). Data are expressed as mean ± SEM. Results were considered significant at $p < 0.05$.

RESULTS

Hemolysis during open TAA / TAAA repair

Plasma levels of fHb and arginase-1, used as markers for hemolysis, were within normal ranges prior to surgery (**Figure 5.1**). After initiation of the ECC, fHb and arginase-1 levels increased and peak plasma levels were found at 2 hours in the reperfusion phase (2h R), indicating ongoing lysis of red blood cells after cessation of ECC. Spearman correlation analysis between areas under the curve of each parameter for every individual patient revealed a significant association between fHb and arginase-1 release ($R_s = 0.83$, $p < 0.01$).

Forearm vascular smooth muscle responses to exogenous NO

Increased plasma concentrations of cell-free hemoglobin may limit NO-mediated vasodilatation. Therefore, we studied FBF in a series of nine consecutive patients undergoing TAAA repair (**Figure 5.2**). Plasma fHb levels were measured in these patients before FBF measurements began (2h R: 20.9 ± 6.5 µmol/l vs. Day 1: 3.8 ± 0.5 µmol/l, $p < 0.05$) and were comparable to those measured in the first series of patients (see **Figure 5.1**).

Figure 5.1 Markers of hemolysis in patients undergoing major cardiovascular surgery. Circulating plasma fHb and arginase-1 during open thoracic - / thoracoabdominal aortic aneurysm repair reached peak values at 2 hours in the reperfusion phase (2h R). Both markers of hemolysis had returned to preoperative range at the first postoperative day. Spearman correlation analysis revealed a significant association between both variables (R_s 0.83, $p < 0.01$). Abbreviations: Op, operation; ECC, extracorporeal circulation; R, reperfusion, * $p < 0.05$; ** $p < 0.01$.

Blood flow responses to SNP, an endothelium-independent NO-donor, were measured postoperatively (2h R) and at day 1 postoperative (Day 1). FBF responses to SNP were significantly reduced direct postoperatively when compared to the first postoperative day ($p < 0.05$; **Figure 5.2A**). Interestingly, using Spearman correlation analysis at 2h R, forearm blood flow was negatively correlated with plasma fHb for both dosages studied (SNP 1 $\mu\text{g}/\text{min}$ Spearman $R_s = -0.89$, $p < 0.01$; SNP 10 $\mu\text{g}/\text{min}$ $R_s = -0.93$, $p < 0.001$, **Figure 5.2B**). To control for vasodilatory capacity the response to exogenous ACh, a vasodilator that induces hyperpolarization of the endothelial smooth muscle cells, was assessed at the same time points. The dose response reaction to ACh was not significantly different at 2h R versus Day 1, indicating unchanged potent vasodilatory capacity (**Figure 5.2C**). All patients were cardiopulmonary stable before, during and after the FBF measurements in which the mean arterial pressure remained between 70 – 90 mmHg, regardless of the dosage SNP or ACh used.

Taken together, these data indicate a deleterious effect of hemolytic product fHb on NO-dependent blood flow responses, whereas NO-independent blood flow responses remained unchanged around cardiovascular surgery with ECC and massive transfusion including cell-salvage.

Figure 5.2 High levels of fHb correlate with an impaired NO-bioactivity *in vivo*. (A) After infusion of the NO-donor sodium nitroprusside (SNP) forearm blood flow (FBF) measurements revealed reduced smooth muscle response at 2 hours reperfusion (2h R), when fHb levels peaked (see Figure 5.1). At the first postoperative day (Day 1) FBF was significantly higher after infusion of SNP, indicating smooth muscle relaxation to exogenous NO infusion was restored. (B) Single exponential decay curve-fit was evaluated by plotting individual FBF responses to SNP at 2h R against plasma fHb levels. Using Spearman correlation test, increasing levels of fHb appeared negatively correlated with FBF responses to SNP for both dosages (SNP 1 µg/min $R_s = -0.89$, $p < 0.01$; SNP 10 µg/min $R_s = -0.93$, $p < 0.001$). Importantly, these findings reproduce data from Reiter *et al.* in 2002, evaluating plasma fHb versus SNP responses in sickle-cell disease patients²⁸. (C) Infusion with Acetylcholine (ACh), which acts predominantly via hyperpolarization induced vasodilatation, at 2h R and Day 1 revealed a similar endothelial smooth muscle reaction to both dosages infused. Basal flow was set at 100 % for every measurement. * $p < 0.05$; ** $p < 0.01$.

In vivo indices of arginase-1 activity

Plasma arginase-1 activity potentially leads to a decreased plasma arginine concentration with a concomitant elevated plasma ornithine concentration. To test for arginase-1 activity, we measured plasma levels of arginine and ornithine. Also, for each individual patient plasma arginine / ornithine ratio was calculated at every time-point (**Figure 5.3**). Plasma arginine and ornithine levels decreased during and after the operation just as several other amino acids (data not shown). Despite increasing plasma levels of arginase-1 (see **Figure 5.1**) no significant changes in plasma arginine / ornithine ratio were observed during the studied period. Moreover, correlation analysis between the increase of plasma arginase-1 and the decrease of plasma arginine levels for each individual patient revealed no association between both parameters (Spearman $R_s = 0.55$; $p = 0.105$). These results do not support increased plasma arginase-1 activity in cardiovascular patients undergoing ECC with massive transfusion including cell-salvage.

Figure 5.3 Plasma amino acid levels. To evaluate plasma arginase-1 activity the substrate plasma arginine (Arg) and product, plasma ornithine (Orn) levels were measured. Arginine / ornithine ratio (Arg / Orn) was calculated to appraise plasma arginase-1 activity. Arg / Orn ratio was not significantly altered during the study, indicating no increased plasma arginase-1 activity. Abbreviations: Op, operation; ECC, extracorporeal circulation; R, reperfusion, h, hours; min, minutes, ** $p < 0.01$.

DISCUSSION

In this study hemolysis, represented by elevated plasma fHb and arginase-1 levels, was observed in cardiovascular patients who underwent ECC and massive transfusion including cell-salvage. NO is the central component in regulation of vasodilatation and maintaining adequate tissue oxygenation and function. Therefore, interference in NO

metabolism, either by scavenging of NO via fHb or by depletion of the NO substrate arginine, may result in vasoconstriction, modulation of platelet activation, enhanced leukocyte adhesion and oxygen free-radical generation in the vascular compartment.¹¹ These disturbances can lead to clinical symptoms such as systemic and pulmonary hypertension, intravascular thrombosis, ischemic cerebrovascular events, priapism and paroxysmal nocturnal hemoglobinuria; all these phenomena are associated with hemolytic crisis in experimental, hereditary or acquired chronic hemolytic diseases.^{12,13}

This study is directed at investigating acute effects of hemolysis during and after surgery. We have now shown that hemolysis, in particular the release of fHb, is associated with an acutely impaired NO-mediated vasodilatory capacity in cardiovascular patients undergoing ECC with massive transfusion and cell-salvage.

Elevated plasma fHb in patients undergoing ECC has been described previously; furthermore, fHb is commonly used as a hemolytic marker when ECC is used. Massive allogeneic transfusion, to cope for considerable blood loss during surgery, contributes to increased levels of plasma fHb, whereas red blood cell damage occurring in cell-salvage devices is also likely to increase levels of circulating plasma fHb.^{4,14} Surprisingly, peak plasma fHb levels were found 2 hours after cessation of the ECC when patients arrived at the Cardiothoracic Intensive Care Unit. Transfusion requirements, both red blood cell and plasma, at that time were minimal and cell-saver blood product had been administered to the patients before transportation to the Cardiothoracic Intensive Care Unit. These data suggest that hemolysis is ongoing after ECC is terminated and the observed increase is a result of exhaustion of natural fHb scavengers such as haptoglobin and CD163.^{15,16} Also, the fact that arginase-1, which has a plasma half-life of 40 minutes¹⁷ increased in this time-period, suggests that ongoing hemolysis explains the increase in fHb.

It is known that fHb can be filtered by the kidney under normal physiologic circumstances, as well as during impaired renal function. Therefore, an effect of renal dysfunction on the clearance of fHb, leading to prolonged elevated fHb levels, cannot be excluded. Meyer *et al.* report in their study that in patients requiring hemodialysis fHb is elevated at baseline before hemodialysis ($12.2 \pm 9.1 \mu\text{M}$) as compared with values reported for control subjects, who are in the nanomolar range.¹⁸ This suggests that in this patient population the renal clearance of fHb is impaired. However, this was measured in patients with end-stage renal failure, whereas in our study no patients were included with end-stage renal disease that needed pre-operative or post-operative dialysis.

Plasma fHb is thought to extravasate into the surrounding extracellular compartment, increasing its potential to interfere with endogenously produced NO thereby further challenging the vasodilatory capacity of vascular smooth muscle cells.¹⁹ Hence, we evaluated whether levels of fHb were correlated with reduced vascular smooth muscle

reaction to exogenous NO. The results of our study show that the responses to the NO-donor SNP were significantly lower when plasma fHb was elevated compared with measurements 24 hours later, when plasma fHb had returned to baseline levels. The observed decreased responses to exogenous NO were strongly correlated with plasma fHb levels in a way similar to what has been reported by Reiter et al. in 2002 evaluating plasma fHb and SNP responses in patients with sickle cell disease. This indicates that across diseases, this process is reproducible.

To test for endothelial dysfunction we evaluated responses to the vasodilator ACh, which acts predominantly via hyperpolarization of endothelial smooth muscle cells resulting in Ca^{2+} influx with a concomitant vasodilatory response, and to a lesser extent via induction of NO synthesis.²⁰ Near equally potent endothelial vasodilatory capacities at both time-points were found after ACh infusion, representing viable endothelial function. Taken together, these data show scavenging of exogenously infused NO (SNP) and support also the general hypothesis of endogenously produced NO-scavenging by fHb.

Arginase-1 release can be the result of both hemolysis and hepatocyte injury. Since the kinetics of arginase-1 release in the present study correlated significantly with the release of fHb, hemolysis appears to be the primary cause of elevated plasma arginase-1. Furthermore, recent data from our group indicate that the activity of plasma arginase-1, at concentrations found in our patients is too low to exert a substantial and lasting effect on plasma arginine, the substrate for NO metabolism.¹⁷ Only plasma arginase-1 levels of 1500 $\mu\text{g/l}$ or higher, as found after deceased donor liver transplantation were able to reduce plasma arginine and increase plasma ornithine levels, potentially hampering NO metabolism.¹⁷ Plasma arginine levels in our patients decreased in parallel with ornithine and other amino acid concentrations as has been described previously after cardiovascular surgery with ECC.²¹ In line, Arg / Orn ratio in the present study remained unchanged. Moreover, we found no association between elevated plasma arginase-1 levels and decreased plasma arginine levels. We conclude therefore, that NO-production in our patient population was not hampered by plasma arginase-1 activity.

Whereas the potential consequences of hemolysis are recently reported for chronic hemolytic diseases, such as sickle cell disease, here we present data that hemolysis is likely to have also consequences for cardiovascular, cardiothoracic and trauma surgery patients. Data from our group show that cardiovascular surgery is associated with intestinal mucosal injury during the post-operative period⁶ as well as acute kidney injury, which are strongly associated with inflammation and worse patient outcome.²² Recently, we found that the total release of plasma fHb was independently correlated with the kidney injury marker N-acetyl glucosaminidase, which in turn was independently associated with the postoperative increase in serum creatinine, used as

marker for acute kidney injury.²³ Further studies are underway to evaluate correlations between perioperative disturbances in vasoregulation and clinical outcome in cardiovascular surgical patients. Also, the potential effect of hemolysis on induction of vascular inflammation, oxidative stress and blood-pressure regulation during elevated levels of circulating fHb remains unclear and need to be addressed in future studies.

In conclusion, our data show diminished vascular smooth muscle reactivity to NO during hemolysis in cardiovascular surgical patients, compromising forearm blood flow, which we attribute to elevated levels of circulating fHb. Such acute disturbances in NO-dependent blood flow potentially contribute to organ injury after cardiovascular surgery. We therefore suggest that either hemolysis should be prevented, or the deleterious effects of fHb should be counteracted in clinical practice to restore NO-dependent blood flow in cardiovascular patients undergoing ECC and massive transfusion including cell-salvage as was recently reviewed.²⁴

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Hemolysis is associated with acute kidney injury during major aortic surgery

Iris C Vermeulen Windsant, Maarten G Snoeijs, Sebastiaan J Hanssen,
Sibel Altintas, John H Heijmans, Thomas A Koeppel,
Geert Willem H Schurink, Wim A Buurman, Michael J Jacobs

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ABSTRACT

Acute kidney injury severely affects patient morbidity and mortality after on-pump cardiovascular surgery. Hemolysis is an inevitable side-effect of cardiopulmonary bypass and results in increased plasma free hemoglobin that may impair tissue perfusion by scavenging nitric oxide. We studied the effect of acute hemolysis on renal injury in 35 patients undergoing on-pump surgical repair of thoracic and thoracoabdominal aortic aneurysms. After surgery, 19 (54%) patients suffered acute kidney injury (AKIN classification). During surgery, plasma free hemoglobin and the tubular injury marker urine N-acetyl- β -D-glucosaminidase increased in patients with and without acute kidney injury, reaching peak levels at 2 hours and 15 minutes after reperfusion, respectively. Furthermore, plasma free hemoglobin was independently correlated with urine N-acetyl- β -D-glucosaminidase ($p = 0.001$), which in turn was independently associated with the postoperative increase in serum creatinine ($p = 0.002$). Importantly, peak plasma free hemoglobin and urine N-acetyl- β -D-glucosaminidase concentrations had significant predictive value for postoperative acute kidney injury (area under the ROC-curve 0.73 and 0.76, $p = 0.04$ and $p = 0.01$ respectively). The association between increased plasma free hemoglobin and renal injury cast new light on the pathophysiology of acute kidney injury and offers a novel therapeutic target to improve clinical outcome after on-pump cardiovascular surgery.

INTRODUCTION

Acute kidney injury (AKI) is an important complication of major cardiovascular surgery that affects 3-48% of patients and is independently associated with mortality.¹⁻³ Currently, specific therapies for postoperative acute kidney injury are not available and patients generally receive supportive care until renal function has recovered spontaneously.⁴ Development of novel interventions to reduce AKI after major cardiovascular surgery requires detailed knowledge of its pathophysiology, which is characterized by a complex interplay of renal vascular dysfunction, tubular epithelial injury and inflammation.⁵

In cardiovascular surgery, the use of cardiopulmonary bypass is associated with acute kidney injury.^{6,7} Hemolysis is a common consequence of cardiopulmonary bypass that is caused by mechanical stress in the perfusion circuit and results in the release of hemoglobin from lysed erythrocytes into the plasma.^{8,9} Cell-free oxyhemoglobin reacts with nitric oxide to form methemoglobin and nitrate. Since nitric oxide is an important vasodilator that plays a central role in blood flow regulation, reduction of nitric oxide bioavailability by free hemoglobin (fHb) may impair tissue perfusion.¹⁰ Indeed, addition of 6 μ M oxyhemoglobin to the perfusion solution of isolated rabbit hearts completely eliminates the activity of endothelium-derived nitric oxide.¹¹ In line with this finding, patients with chronic hemolysis due to sickle cell disease are characterized by an attenuated rise in forearm blood flow after infusion of the nitric oxide donor sodium nitroprusside as compared to healthy volunteers; forearm blood flow response was reduced by 80% in patients with plasma fHb concentrations over 6 μ M.^{10,12} In addition, after glomerular ultrafiltration, urine fHb may cause acute kidney injury by generating reactive oxygen species and by aggregating into casts in the tubular lumen.^{13,14}

Until now, human studies have focused on the association between urinary free hemoglobin and acute kidney injury.¹³ The vasoconstrictive effects of fHb in plasma may provide an additional explanation for hemolysis-induced renal injury. In the current study, we show that increased plasma fHb concentrations are independently associated with injury to the proximal tubular epithelium during open surgical repair of thoracic and thoracoabdominal aortic aneurysms with cardiopulmonary bypass. Moreover, plasma fHb levels 2 hours after surgery predicted acute kidney injury in the early postoperative period. This finding casts new light on the pathophysiology of AKI and offers a novel therapeutic target to improve clinical outcome after major cardiovascular surgery.

RESULTS

Open repair of thoracic and thoracoabdominal aortic aneurysms with cardiopulmonary bypass causes AKI

We studied 35 consecutive patients undergoing open repair of a thoracic aortic aneurysm (N = 1) or thoracoabdominal aortic aneurysm (N = 34) with cardiopulmonary bypass. Clinical data are presented in Table 6.1. None of the patients were diagnosed with preoperative hemolytic disease. Overall in-hospital mortality was 20% (N = 7), there were no intraoperative deaths. Nineteen patients (54%) developed postoperative AKI (**Table 6.1**) as defined by the AKIN classification. Mortality was numerically higher in AKI patients compared to patients without AKI (32%, N = 6; vs. 6%, N = 1), but this difference did not reach statistical significance ($p = 0.10$). Renal function fully recovered during hospitalization in 85% (N = 11 out of 13) of surviving AKI patients. In the two remaining AKI patients, renal function improved from Stage 3 to Stage 2. The impact of AKI on overall patient outcome was reflected by a trend towards longer hospitalization times (21 (13-34) days vs. 14 (12-16) days, $p = 0.05$), and ICU-admission times (7 (4-28) days vs. 4 (3-7) days, $p = 0.11$) in AKI vs. non-AKI patients, respectively. Baseline renal function (glomerular filtration rate (eGFR) estimated by the abbreviated Modification of Diet in Renal Disease (MDRD) equation¹⁵) did not differ between patients with or without postoperative AKI (95 ± 43 and 77 ± 30 ml/min/1.73 m² respectively, $p = 0.28$).

Open repair of thoracic and thoracoabdominal aortic aneurysms results in hemolysis

To study the development of hemolysis during surgery, plasma fHb levels were analyzed at 8 preset perioperative time-points from preoperative until the second postoperative day. Preoperative fHb levels were comparable to values measured in healthy subjects (< 4 μ M). At the end of cardiopulmonary bypass, plasma fHb levels had risen significantly in AKI patients ($p < 0.05$) compared to baseline values (Figure 6.1A). Plasma fHb values further increased in both groups in the first few hours after cessation of cardiopulmonary bypass ($p < 0.001$) and remained elevated above the preoperative level during the first two postoperative days. Comparison of plasma fHb levels between patients with and without postoperative acute kidney injury (Figure 6.1B) showed that at two hours after reperfusion plasma fHb was significantly higher in AKI patients (16.6 ± 12.4 μ M vs. 7.9 ± 4.2 μ M, $p < 0.05$). Overall, plasma fHb concentrations of 6 μ M or more, levels associated with a reduction in microcirculatory blood flow, were measured in 83% of patients at any time point during the study period. Urine fHb was undetectable (< 2 μ M) in all patients, suggesting that glomerular

filtration of fHb is unlikely to be a major contributor to acute kidney injury after cardiovascular surgery.

Table 6.1 Characteristics of patients undergoing open surgical repair for thoracic or thoracoabdominal aortic aneurysms with cardiopulmonary bypass.

Preoperative variables*	Non-AKI (N = 16)	AKI (N = 19)	p-value
Age (years), <i>median</i> (IQR)	58 (54-66)	68 (56-72)	0.14
Male, <i>n</i> (%)	9 (56)	14 (74)	0.31
Indication <i>n</i> (%)	-	-	0.04
TAA	1 (6)	0 (0)	
TAAA type I [†]	2 (13)	1 (5)	
TAAA type II [†]	2 (13)	9 (47)	
TAAA type III [†]	7 (44)	5 (21)	
TAAA type IV [†]	1 (6)	0 (0)	
TAAA type V [†]	3 (19)	5 (26)	
Concomitant aortic disease, <i>n</i> (%)	-	-	0.50
Type B-dissection (Stanford classification)	2 (13)	4 (21)	
Marfan disease	2 (13)	1 (5)	
Aortic stenosis (high grade)	2 (13)	0 (0)	
eGFR (ml/min/1.73 m ²) [#] , <i>mean</i> ± s.d.	77 ± 30	95 ± 43	0.28
Chronic Kidney Disease (eGFR<60), <i>n</i> (%)	8 (28)	9 (31)	0.70
Intraoperative variables*			
Selective organ perfusion, <i>n</i> (%)	14 (88)	19 (100)	0.20
Total operation time (min), <i>mean</i> ± s.d.	369 ± 92	433 ± 96	0.06
Cardiopulmonary bypass time (min), <i>mean</i> ± s.d.	163 ± 56	195 ± 64	0.14
Aortic cross clamp time (min), <i>mean</i> ± s.d.	114 ± 58	144 ± 71	0.21
Blood loss (L), <i>median</i> (IQR)	8 (5-25)	8 (6-21)	0.86
Packed cell transfusion (nx350 ml units), <i>median</i> (IQR)	10 (7-16)	10 (7-18)	0.95
Postoperative variables*			
In hospital mortality, <i>n</i> (%)	1 (6)	6 (31)	0.10
Total intensive care unit stay (days), <i>median</i> (IQR)	4 (3-7)	7 (4-28)	0.11
Total hospitalization time (days), <i>median</i> (IQR)	14 (12-16)	21 (13-34)	0.06
Creatinine change < 48h (%), <i>median</i> (IQR)	115 (93-129)	243 (166-300)	<.001
Acute kidney injury (Stage 1, 2, or 3 of AKIN-classification), <i>n</i> (%)	-	19 (100)	-
Stage 1	-	6 (32)	-
Stage 2	-	4 (21)	-
Stage 3	-	9 (47)	-
Dialysis	-	7 (37)	-

Abbreviations: IQR, interquartile range from the 25th until 75th percentile; TAA, thoracic aortic aneurysm, TAAA, thoracoabdominal aortic aneurysm; eGFR, estimated glomerular filtration rate; AKIN, Acute Kidney Injury Network. [†] Roman numerals refer to the TAAA classification system by Crawford³⁷; see also Methods [#] Glomerular filtration rate was estimated by the abbreviated Modification of Diet in Renal Disease (MDRD) as recommended by the National Kidney Foundation¹⁵.

Figure 6.1 Hemolysis (A) and renal proximal tubular epithelial damage (B) during open thoracic or thoracoabdominal aortic repair in patients with (N = 19) and without (N = 16) acute kidney injury. Urinary NAG concentrations were corrected for urine creatinine values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to preoperative levels. # $p < 0.05$ between AKI and non-AKI patients.

Abbreviations: NAG, N-acetyl- β -D-glucosaminidase; AKI, acute kidney injury; fHb, free hemoglobin; CPB, cardiopulmonary bypass; R, reperfusion; min, minutes; h, hours.

Hemolysis is correlated with renal tubular injury during open repair of thoracic and thoracoabdominal aortic aneurysms

To study renal tubular injury during surgery, urinary N-acetyl-beta-(D)-glucosaminidase (NAG) concentration was measured and corrected for urinary creatinine. NAG is a lysosomal brush border enzyme present in proximal tubular epithelial cells that are particularly vulnerable to ischemic injury.¹⁶ Parallel to the development of hemolysis, a significant increase in urine NAG was observed during cardiopulmonary bypass (**Figure 6.1B**). NAG levels remained elevated above preoperative levels until the second postoperative day. Moreover, when comparing patients with and without AKI,

significantly higher urine NAG levels were detected in patients with AKI at 15 minutes after reperfusion (39.6 ± 45.0 U/mmol creatinine vs. 12.5 ± 7.7 U/mmol creatinine, $p < 0.05$).

Next, we analyzed the association between hemolysis and renal tubular injury. To this end, the area under the curve of fHb (AUC_{fHb}) and NAG (AUC_{NAG}) was calculated, reflecting total fHb release and NAG excretion during the study period. AUC_{fHb} and AUC_{NAG} showed a strong positive correlation (Figure 6.2, Pearson $r = 0.75$, $p < 0.0001$). This correlation remained when the subgroups of AKI and non-AKI patients were analyzed separately (Pearson $r = 0.79$, $p < 0.001$, and $r = 0.64$, $p < 0.01$, respectively). Finally, AUC_{fHb} remained significantly associated with AUC_{NAG} ($p = 0.001$, **Table 6.2**) after adjustment for cardiopulmonary bypass time – a potential confounder – in multivariable linear regression.

Table 6.2 Multivariable linear regression analysis for AUC_{NAG}

Variable	Beta	95% CI	p-value
Cardiopulmonary bypass time (min)	0.11	-2.30 - 2.51	0.93
AUC_{fHb}	1.31	0.57 - 2.05	0.001

Abbreviations: AUC, area under the curve; NAG, N-acetyl- β -D-glucosaminidase; fHb, plasma free hemoglobin; CI, 95% confidence interval.

Hemolysis and renal tubular injury are associated with postoperative renal dysfunction

To assess whether intraoperative renal tubular injury was associated with postoperative AKI, we analyzed the relation between NAG levels (at 15 minutes reperfusion) and perioperative serum creatinine changes (highest value reached within the first two postoperative days as a percentage from baseline). Using multivariable linear regression we studied the association of NAG with creatinine rise independently of 3 established risk factors for AKI; preoperative age, preoperative eGFR and intraoperative cardiopulmonary bypass time. NAG levels ($p = 0.002$), preoperative eGFR ($p = 0.03$), and age ($p = 0.01$) were significantly associated with serum creatinine increases (**Table 6.3**).

To study the prognostic value of plasma fHb and urine NAG for postoperative AKI, we analyzed the sensitivity and specificity of peak plasma fHb and urine NAG using receiver operating characteristic (ROC) curves (**Figure 6.3**). Peak fHb and NAG values were observed at two hours reperfusion and at 15 minutes reperfusion, respectively. Peak plasma fHb and urine NAG both held significant diagnostic power for the prediction of acute kidney injury (AUC 0.73, $p = 0.04$ and AUC 0.76, $p = 0.01$, respectively). Optimal cut-off values to discriminate between patients with and without AKI were 10 μ M for plasma fHb (sensitivity: 79%, specificity: 69%) and

14 U/mmol creatinine for urinary NAG (sensitivity: 73%, specificity: 72%). This indicates that it is possible to differentiate between patients with and without postoperative AKI based on plasma fHb levels shortly after surgery.

Table 6.3 **Multivariable linear regression analysis for perioperative creatinine change***

Variable	Beta	95% CI	P -value
Age (years)	3.34	0.97 - 5.71	0.01
Preoperative eGFR (ml/min/1.73m ²)	0.91	0.10 - 1.73	0.03
Cardiopulmonary bypass time (min)	-0.01	-0.54 - 0.52	0.97
NAG _{15min R} (U/mmol creatinine)	1.53	0.61 - 2.46	0.002

*Creatinine change expressed as change from baseline to highest value reached during the first two postoperative days in %. Abbreviations: eGFR, estimated glomerular filtration rate; NAG, N-acetyl-β-D-glucosaminidase; 15min R, 15 minutes after reperfusion; CI, 95% confidence interval

Figure 6.3 **Receiver Operating Characteristics (ROC) Curve presenting sensitivity and 1 - specificity for peak plasma fHb levels at 2 hours reperfusion (black line) and peak urine NAG levels at 15 minutes reperfusion (dotted line).** Abbreviations: AUC, area under the receiver operating characteristics curve; NAG, N-acetyl-β-D-glucosaminidase; fHb, free hemoglobin.

DISCUSSION

Acute kidney injury is a common complication after surgery using cardiopulmonary bypass with a profound impact on postoperative morbidity and mortality.¹⁷ Despite major improvements in biocompatibility and flow performance of the bypass circuits, the incidence of AKI after cardiovascular surgery has not decreased significantly over the past decade.¹⁸ Therefore, development of novel interventions to reduce AKI after major cardiovascular surgery requires further study of its pathophysiology.

In the current study, we show that the concentrations of plasma fHb and urine NAG increased during on-pump repair of thoracic and thoracoabdominal aortic aneurysms, which indicates that hemolysis and tubular epithelial injury occurred. Hemolysis during cardiac surgery has previously been reported and is considered to be caused by cardiopulmonary bypass circuits, transfusion of stored red blood cells, and cell salvage devices.^{8,19-21} Interestingly, we found that the total release of plasma fHb was independently correlated with urine NAG, which in turn was independently associated with the postoperative increase in serum creatinine. This clinical observation is supported by rodent experiments in which intravenous infusion of fHb increased urine NAG concentrations 10-fold while decreasing glomerular filtration rate by 30%.²² Furthermore, scavenging of plasma fHb by infusion of haptoglobin in patients undergoing cardiac surgery was associated with a 4-fold reduction of urine NAG as compared to a historical cohort without haptoglobin infusion.²³ In addition, cardiac surgery without cardiopulmonary bypass, which is expected to attenuate intravascular hemolysis, causes a significant reduction of postoperative acute kidney injury as compared to on-pump surgery.²⁴ In our study, peak plasma fHb levels of 10 μ M or higher held a significant diagnostic value for the prediction of AKI. The sensitivity and specificity of this marker to predict AKI in clinical practice should be validated in future studies. Taken together, these findings indicate that plasma free hemoglobin is a risk factor for tubular epithelial damage and AKI after cardiovascular surgery.

The diagnosis of AKI using serum creatinine is difficult, since serum creatinine is a poor marker of early renal dysfunction in the non-steady state of the surgical patient. Since the impact of AKI on the outcome of the patient is profound, the quest for sensitive and specific biomarkers has been designated as priority. During the past decade several candidate biomarkers (such as neutrophil gelatinase-associated lipocalin, kidney injury molecule-1 and interleukin-18) were reported, however none has been generally accepted for clinical use. The diagnostic accuracy of urine NAG and plasma fHb in the current study might provide a valuable tool for early detection of postoperative AKI, enabling timely therapeutic interventions.²⁵ Nevertheless, sensitivity and specificity may be overestimated when calculated with the same data set that has been used to determine the optimal cut-off point for a diagnostic test. Therefore, the diagnostic accuracy of our suggested cut-off point for plasma fHb should be validated in future studies.

The observed relation between *plasma* fHb and renal damage in this human setting is important since earlier studies on hemolysis-induced AKI focussed traditionally on the role of filtered fHb in urine.^{13,26,27} Indeed, urine fHb and its catabolic products iron and heme catalyze the generation of reactive oxygen species that damage the tubular epithelium when present in the glomerular ultrafiltrate.¹⁴ In support, administration of the iron scavenger deferoxamine attenuated the glomerular and tubular dysfunction

induced by intravenous administration of fHb in rats.²⁸ Similarly, reduced plasma ferritin concentrations – which may be associated with reduced iron scavenging – were associated with increased AKI after human cardiovascular surgery.²¹ Second, fHb may precipitate in the acidic ultrafiltrate, forming casts that obstruct the tubular lumen and reduce glomerular filtration.^{14,29} Prevention of cast formation by alkalinization of the urine reduced tubular injury and glomerular dysfunction after intravenous fHb infusion in rats.¹⁴ Since fHb was not detected in urine samples of our patients, we propose that glomerular filtration of fHb is unlikely to be a major contributor to AKI.

An additional and complementary explanation for the induction of renal injury by hemolysis is provided by the recent discovery that plasma oxy-fHb converts the vasodilator nitric oxide into biologically inactive nitrate.¹⁰ Since nitric oxide plays a central role in blood flow regulation, reduction of nitric oxide bioavailability by fHb may impair tissue perfusion and induce AKI. In line, induction of hemolysis by water infusion or direct administration of fHb in dogs increased plasma nitric oxide consumption and resulted in elevated systemic vascular resistance and reduced creatinine clearance. These hemodynamic effects of acute hemolysis were attenuated by nitric oxide inhalation which converts oxy-fHb into biologically inactive methemoglobin.¹² Furthermore, in patients with chronic hemolytic disorders such as sickle cell disease and malaria, the extent of hemolysis was correlated with endothelial dysfunction, pulmonary hypertension and coagulation.³⁰⁻³²

In the light of these findings, we propose that the association between acute hemolysis and renal injury in patients undergoing on-pump cardiovascular surgery may be caused by scavenging of nitric oxide through fHb. First, plasma fHb levels in our study are comparable to concentrations reported to reduce blood flow, nitric oxide bioavailability and impair renal function in animal experiments and men.^{10,12} Second, in patients after on-pump surgical repair of thoracic and thoracoabdominal aortic aneurysms, we found that the increase in forearm blood flow after infusion of the nitric oxide donor sodium nitroprusside was significantly lower at the time of peak plasma fHb concentration as compared to the increase at one day after surgery. Since the vascular response to acetylcholine – with vasodilatory effects that are largely independent of nitric oxide – was similar at these time points, the impaired reactivity to sodium nitroprusside is most likely not caused by vascular dysfunction but rather by reduced bioavailability of the administered nitric oxide donor (Hanssen *et al.*, Thorac Cardiovasc Surg 2012, in press). These data, although providing indirect proof, support our findings and suggest that scavenging of nitric oxide by plasma fHb may contribute to the association between acute hemolysis and renal injury.

The major strength of the current study is its translation of experimental findings on the effects of hemolysis on renal function into the clinical setting of on-pump cardiovascular surgery. Patients undergoing open repair of thoracic and thoraco-

abdominal aortic aneurysms are particularly suitable for clinical investigation of the pathophysiology of AKI since these individuals are at exceptionally high risk of postoperative renal dysfunction. The strong and significant correlations between hemolysis and renal injury in our patients should not be interpreted as causal relationships. The results from the current study ask for further analysis in larger patient groups, and interventional studies using haptoglobin infusion or nitric oxide inhalation to inactivate fHb or using nitrite administration to increase nitric oxide bioavailability may provide further support for a causal relationship between nitric oxide scavenging by plasma fHb and AKI after on-pump cardiovascular surgery.^{12,23,33,34} The pathophysiology of AKI is highly complex and involves multiple factors other than intravascular hemolysis such as ischemia and reperfusion, hemodilution, inflammation and baseline renal function.⁵ The current study provides additional insights into the pathophysiology of this condition relevant for the design of multivariable risk scores and future therapeutic studies.

In conclusion, we show that increased plasma fHb concentrations are independently associated with injury to the proximal tubular epithelium during surgery with cardiopulmonary bypass. Peak plasma fHb concentrations 2 hours after surgery were predictive of AKI within the first postoperative days. This finding casts new light on the pathophysiology of AKI during on-pump cardiovascular surgery that may also play a role in other conditions associated with acute hemolysis such as hemodialysis and major trauma.^{35,36}

METHODS

Patients

Patients admitted to Maastricht University Medical Center or University Hospital Aachen between May 2006 and November 2008 to undergo elective open repair of a thoracic aortic aneurysm or thoracoabdominal aortic aneurysm with cardiopulmonary bypass were included. The study was approved by the Institutional Review Board of both institutes and written informed consent was obtained from every patient prior to surgery. Patients requiring preoperative dialysis due to pre-existent renal failure were excluded. Preoperative clinical data were prospectively collected from the patient's medical record. Glomerular filtration rate (eGFR) was estimated by the abbreviated Modification of Diet in Renal Disease (MDRD) as recommended by the National Kidney Foundation.¹⁵ Thoracoabdominal aortic aneurysm type was classified according to Crawford's classification. Type I thoracoabdominal aortic aneurysms extend distally from the left subclavian artery, in any case above the 6th intercostal space, down to encompass the aorta at the origin of the celiac trunk and superior mesenteric artery.

The renal arteries may also be involved, but the aneurysm does not extend into the infrarenal segment. Type II thoracoabdominal aortic aneurysms extend distally from the left subclavian artery until the aortic bifurcation. Type III thoracoabdominal aortic aneurysms begin in the distal half of the descending thoracic aorta, below the sixth intercostal space, and extend to the aortic bifurcation. Extent IV aneurysms involve the entire abdominal aorta from the diaphragm to the bifurcation. Type V thoracoabdominal aortic aneurysms originate from the lower thoracic aorta to the aortic section just below the visceral arteries.³⁷

Surgical procedure

An indwelling radial artery catheter was placed as part of standard anesthetic care. After induction, patients were placed in a left helical position. Thoracolaparotomy in type I, II, and III type thoracoabdominal aortic aneurysms was performed through the sixth intercostal space. In type IV thoracoabdominal aortic aneurysms a small thoracolaparotomy through the eighth intercostal space sufficed. In patients with an isolated thoracic aneurysm, a small laparotomy was performed. Surgical repair was typically done from proximal to distal. After heparinization (0.5 mg/kg), cardiopulmonary bypass was established using a centrifugal pump with membrane oxygenation (Jostra Rotaflow with Jostra Quadrox, Maquet Cardiopulmonary AG, Hirrlingen, Germany). Depending on the extent of the aneurysm, cardiopulmonary bypass was expanded with a 4-branched tubing system enabling pressure-controlled perfusion with oxygenated blood of the celiac trunk, superior mesenteric artery, renal arteries, and if necessary intercostal arteries, during aortic cross clamping (ACC). During ACC, renal blood flow and perfusion pressure was closely monitored and kept at 200-280 ml/min, and 60-70 mmHg, respectively. A cell-salvage device (CATS, Fresenius SE, Bad Homburg, Germany) was used in every patient. The exact surgical protocol was identical in both institutions and has been described in detail elsewhere.^{38,39} Postoperatively, all patients were transported to the Cardiothoracic Intensive Care Unit for postoperative monitoring, circulatory and ventilatory support.

Preoperative kidney dysfunction and postoperative acute kidney injury

All individuals with a GFR <60 ml/min/1.73 m², irrespective of the presence or absence of kidney damage, were classified as having chronic kidney disease (CKD) in accordance with the National Kidney Foundation Guidelines.⁴⁰ Postoperative Acute Kidney Injury (AKI) was defined according to the AKI Network (AKIN) classification (**Table 6.1**).⁴¹ The AKIN classification provides three grades of postoperative AKI of increasing severity (Stage 1, Stage 2, and Stage 3) and is based on relative changes in either serum creatinine or urine output (whichever is worst) compared to baseline (preoperative) levels. All patients requiring (transient) postoperative dialysis were classified as Stage 3

AKI, irrespective of creatinine change. We stratified patients into the AKIN stages based on creatinine changes due to the fact that urine output data was not sufficiently recorded in all patients.

Blood sampling, urine sampling and sample processing

Arterial blood was drawn preoperatively and at 8 pre-set perioperative time points; T1, preoperative; T2, before start cardiopulmonary bypass; T3, end cardiopulmonary bypass; T4, 15 minutes reperfusion (15 minutes after cessation of cardiopulmonary bypass); T5, 2 hours reperfusion; T6, 6 hours reperfusion; T7, Day 1 postoperatively; T8, Day 2 postoperatively. Whole blood was collected in EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ). Simultaneously a fresh spot urine sample was taken from the bladder catheter. Whole blood and urine samples were immediately put on ice and centrifuged within 15 minutes after collection (1500 g at 4°C for 15 min), aliquoted and stored at -80°C until further analysis.

Laboratory analysis of hemolysis and renal tubular damage

fHb concentrations, indicating hemolysis, were measured in all patients (N = 35) by derivative spectrometry as described elsewhere.⁴² The detection limit of the assay was 2 µM.

To assess renal tubular damage, urinary NAG concentrations were determined by means of an enzyme colorimetric assay according to manufacturer's instructions (HaemoScan, Groningen, the Netherlands). NAG is excreted predominantly by the proximal renal tubular cells. Due to its relatively large molecular weight (>130 kD), high levels of urinary NAG preclude glomerular filtration. Therefore, increased levels of NAG imply renal tubular damage. Furthermore, NAG is not reabsorbed by the tubules.⁴³ Results were normalized to urinary creatinine values and expressed as U/mmol creatinine.

Statistics

Continuous data were presented as median (interquartile range; IQR from the 25th-75th percentile) or mean $\pm$ s.d, depending on Gaussian distribution (checked using histograms and normal Q-Q plots), and as N (%) for dichotomous data. Differences in patient characteristics between study groups were compared using Pearson Chi-square test with Fisher's correction, when appropriate, for dichotomous variables. Continuous data were analyzed using independent sample T-test or Mann-Whitney-U test depending on Gaussian distribution. Subsequently, changes in fHb and NAG levels compared to preoperative values were tested using the paired T-test. To characterize the total amount of hemolysis and tubular injury during the study period, the area under the curve (AUC) for log-normalized fHb (AUC_{fHb}) and NAG (AUC_{NAG}) was

calculated for each patient using trapezoidal analysis with time as a baseline. Subsequently, the correlation between hemolysis and tubular injury was analyzed using Pearson correlation in the total patient population ($N = 35$) and subgroups of AKI and non-AKI. To correct for other risk factors, the relation between AUC_{NAG} and AUC_{fHb} , and the relation between NAG values and changes in perioperative serum creatinine levels was assessed by multivariable linear regression analysis (raw data were used). Based on the number of included patients, we could adjust for 3 other covariates in the latter multivariable linear regression analysis. In order to analyze the predictive value of plasma free hemoglobin and urinary NAG for AKI, receiver operating characteristics (ROC) curves were drawn by plotting sensitivity versus against $1 - \text{specificity}$ for all possible thresholds. Overall accuracy of the markers in detecting AKI was represented by area under the curve (AUC). Best cutoff points were defined as the maximum sum of sensitivity and specificity. Statistical calculations were made using SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL), and Prism 4.03 for Windows (GraphPad Software Inc. San Diego, CA). A p-value <0.05 was considered to indicate statistical significance.

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Hemolysis increases nitric oxide consumption and visceral tissue damage after cardiac surgery

Iris C Vermeulen Windsant, Norbert CJ de Wit, Jonas TC Sertorio,
Annemarie M van Bijnen, Yuri M Ganushchak, John H Heijmans,
Jose E Tanus – Santos, Michael J Jacobs, Jos G Maessen, Wim A Buurman

Submitted

ABSTRACT

Background

Visceral injury and AKI negatively impact patient outcome after cardiac surgery. Enhanced nitric oxide (NO) consumption, leading to reduced NO-bioavailability, as a consequence of cardiopulmonary bypass-induced hemolysis, may play an important pathophysiological role in this setting.

Methods and results

Hemolysis (by plasma free hemoglobin; fHb), plasma NO-consumption, haptoglobin (Hp), renal tubular injury (using urinary N-Acetyl- β -D-glucosaminidase; NAG), intestinal mucosal injury (through plasma intestinal fatty acid binding protein; IFABP), and AKI were studied in patients undergoing off-pump cardiac surgery (OPCAB, N = 7), on-pump coronary artery bypass grafting (CABG, N = 30), or CABG+Valve surgery (N = 30). fHb and NO-consumption significantly increased, while Hp decreased significantly, in CABG and CABG+Valve patients ($p < 0.0001$). The extent of hemolysis and NO-consumption correlated significantly ($r^2 = 0.75$, $p < 0.0001$). Also, NAG and IFABP increased in both groups ($p < 0.0001$, and $p < 0.001$, respectively) indicating significant visceral tissue injury, and both were significantly associated with hemolysis ($R_s = 0.70$, $p < 0.0001$, and $R_s = 0.26$, $p = 0.04$, respectively) and NO-consumption ($R_s = 0.55$, $p = 0.002$, and $R_s = 0.41$, $p = 0.03$, respectively), also after multivariable logistic regression analysis. OPCAB patients did neither show increased fHb, enhanced NO-consumption, nor visceral tissue damage or AKI. Patients suffering from AKI (N = 9, 13.4%) displayed significantly higher fHb and NAG levels already during surgery compared to non-AKI patients.

Conclusions

This study shows that hemolysis is an independent risk factor for visceral injury during cardiac surgery by limiting NO-bioavailability. This observation offers a novel diagnostic and therapeutic target to improve outcome after cardiothoracic surgery.

INTRODUCTION

Cardiac surgery has become common practice world-wide. Nevertheless, the more complex cardiac procedures are still associated with a relatively high incidence of postoperative complications. Visceral complications, such as renal tubular damage and loss of intestinal wall integrity during surgery, significantly contribute to increased morbidity and mortality in this setting.^{1,2} Also, acute kidney injury (AKI) is a common and serious postoperative complication with incidence rates varying between 5% - 30%.^{3,4} AKI-associated morbidity and mortality is high, especially when patients require dialysis.⁵

The use of cardiopulmonary bypass (CPB) contributes to the development of renal tubular damage and intestinal mucosal injury through induction of (microcirculatory) blood flow alterations, ischemia-reperfusion injury, hemodilution, and a pro-inflammatory response.⁶⁻⁸ Hemolysis is another common consequence of CPB and results in the circulatory release of cell free hemoglobin (fHb).⁹ The recent discovery of the potent nitric oxide (NO) scavenging property of fHb has been associated with systemic and pulmonary hypertension, decreased organ perfusion, and increased mortality during exacerbations of diseases characterized by chronic hemolysis.^{10,11} Recently, it has been shown that also acute increases of plasma fHb during hemodialysis lead to intravascular NO depletion, and impaired vascular function in patients.¹² These results substantiated our previous findings that acutely enhanced plasma fHb concentrations were independently associated with renal proximal tubular injury and postoperative AKI after CPB-assisted major aortic surgery.¹³

The identification of risk factors for the development of visceral injury during cardiac surgery is imperative for the development of specific treatment strategies to improve patient outcome. In the present study we aimed to test the hypothesis that the development of hemolysis during cardiac surgery is principally a consequence of CPB, leads to increased intravascular consumption of NO (reflecting decreased NO-bioavailability¹⁰), and contributes to the development of both visceral tissue damage during surgery, and to postoperative renal dysfunction. In order to investigate the impact of CPB use on the development of intraoperative hemolysis, we studied changes of fHb, haptoglobin (the physiologic intravascular fHb scavenger), plasma NO-consumption, and markers of renal tubular damage and intestinal mucosal injury in three groups of patients undergoing cardiac surgery associated with increasing perfusion times: off-pump coronary artery bypass grafting CABG (OPCAB), CABG with CPB, and combined CABG and valve surgery with CPB (CABG+Valve).

METHODS

Patients

Based on a pilot study, power analysis indicated that inclusion of 30 patients per study group enabled detection of statistical significant differences in fHb levels. As we hypothesized that the duration of cardiopulmonary bypass time influences the extent of hemolysis, we included 30 consecutive adult patients undergoing elective on-pump CABG surgery and 30 consecutive patients undergoing elective CABG+Valve surgery (reconstruction and/or replacement of any valve), meeting the inclusion and exclusion criteria. Patients undergoing OPCAB surgery were included to serve as a control group as we expected that hemolysis does not develop in this patient group. However, as OPCAB surgery is infrequently performed at our institution, we included all OPCAB patients meeting the inclusion and exclusion criteria during the inclusion period of the CABG and CABG+Valve patients. This resulted in the final inclusion of 7 OPCAB patients. In total, sixty-seven adult patients undergoing elective cardiac surgery at the Department of Cardiothoracic Surgery of the Maastricht University Medical Center+ between November 2009 and January 2011 were studied. The study was approved by the Institutional Review Board and written informed consent was obtained from every patient prior to surgery. Exclusion criteria were preoperative estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 m², diabetes as these patients display higher baseline levels of N-Acetyl- β -D-glucosaminidase (NAG), or presence of chronic hemolytic disease. The cardiac surgery protocol as performed at our institution has been described in more detail elsewhere.¹⁴

Study endpoints

The primary study endpoint was to investigate whether the extent of hemolysis, reflected by plasma fHb concentrations, is able to cause a significant increase in intravascular NO-consumption during cardiac surgery, thus impairing NO-bioavailability. Second, we evaluated the extent of visceral tissue injury which develops during OPCAB surgery, CABG surgery and CABG+Valve surgery. In addition, the correlation between increased plasma fHb levels, increased NO-consumption, visceral tissue damage as indicated by increased urinary and plasma levels of renal and intestinal damage, respectively, and postoperative AKI was investigated. Lastly, we investigated circulatory levels of Haptoglobin, an important fHb binding protein involved in clearance of fHb.

Definition of postoperative acute kidney injury

Postoperative AKI was defined according to the AKI Network (AKIN) classification using serum creatinine changes within the first 2 postoperative days.¹⁵ The AKIN

classification provides three grades of postoperative AKI of increasing severity (Stage 1, Stage 2, and Stage 3). Patients requiring (transient) postoperative dialysis were classified as Stage 3, irrespective of creatinine change.

Blood sampling, urine sampling, and sample processing

Arterial blood and a fresh spot urine sample were obtained at 8 pre-set perioperative time points; 1, preoperatively, after induction but prior to sternotomy; 2, before start CPB; 3, end CPB; 4, 15 minutes reperfusion (15 minutes after cessation of CPB); 5, 2 hours reperfusion; 6, 4 hours reperfusion; 7, Day 1 postoperatively; 8, Day 2 postoperatively. Whole blood was collected in EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ). All samples were immediately put on ice and centrifuged within 15 minutes after collection (1500g at 4°C for 15 min without brake), aliquoted, and stored at -80°C until further analysis.

Analysis of hemolysis, haptoglobin, renal tubular damage, and intestinal damage

Plasma fHb concentrations were measured by derivative spectrometry as previously described.¹⁶ The lower detection limit of the assay was 2 μ M. Since intravascular hemolysis can lead to consumption of plasma haptoglobin (Hp), the physiological fHb-scavenger¹⁷, the course of plasma Hp during surgery was also studied. Plasma Hp was measured on a validated Beckman LX20 clinical chemistry analyzer (Beckman Coulter, Brea, CA) via a turbidimetric method by the Central Diagnostic Laboratory of the Maastricht University Medical Center. To assess renal tubular damage, urinary NAG concentrations were determined by an enzyme colorimetric assay according to the manufacturer's instructions (HaemoScan, Groningen, The Netherlands). NAG is excreted predominantly by the proximal renal tubular cells. Owing to its relatively large molecular weight (4130 kDa), high levels of urinary NAG preclude glomerular filtration. Furthermore, NAG is not reabsorbed by the tubules. Therefore, increased urinary levels of NAG imply renal tubular damage.¹⁸ Results were normalized to urinary creatinine to correct for dilution and expressed as U/mmol creatinine. Intestinal mucosal damage was studied using plasma concentrations of intestinal fatty acid binding protein (IFABP) which were assessed by an in-house human IFABP ELISA with a detection limit of 12.5 pg/mL. IFABP is a small (15 kDa) cytosolic protein exclusively expressed by mature enterocytes, and is rapidly released into the circulation upon enterocyte damage where it can be readily measured. IFABP is an early, sensitive, and specific marker of clinically relevant intestinal mucosal damage.¹⁹

As the extent of hemodilution during surgery differed between the three study groups, impairing optimal comparison of the studied plasma proteins fHb, Hp, and IFABP, we corrected these values for plasma hematocrit at the moment of blood sampling.

NO consumption assay

To evaluate the NO-consuming capacity of plasma by fHb, we randomly selected 29 patients (5 OPCAB, 12 CABG, and 12 CABG+Valve patients) using SPSS. The complete NO consumption protocol is described elsewhere.^{10,11} Briefly, a 40 μ M solution of the NO-donor, DETA NONOate (Cayman Chemical, Ann Harbor, MI) was prepared in PBS (pH 7.4) in a glass vessel purged with nitrogen in-line with a NO chemiluminescence analyzer (Sievers Model 280i, GE, Boulder, CO). The subsequent decay of DETA NONOate, releasing NO, produced a steady state NO signal of about 50-70 mV. When the signal became stable, 50 μ L of plasma was injected into the DETA NONOate solution, decreasing the NO signal in case of NO-consumption. Data were analyzed with the software program ORIGIN Version 6.1 (OriginLab, Northampton, MA) for analysis of the area under the curve (AUC) of decreasing NO-signal over time. The amount of NO consumption by plasma was quantified by comparison of the AUC with that of NO gas standards (produced from injections of nitrite into triiodide).

Statistics

Continuous data are presented as median and interquartile range (IQR, 25th-75th percentile) or mean $\pm$ SEM, depending on Gaussian distribution (checked using histograms and normal Q-Q plots). Dichotomous data are depicted as % (N). Differences between study groups were compared using Pearson Chi-square test with Fisher's correction when appropriate (dichotomous variables), or independent sample T-test or Kruskal-Wallis test (for continuous variables). Overall changes in fHb, NO-consumption, Hp, NAG, and IFABP levels within groups were tested using the Friedman test for repeated data. If this test yielded a significant result, a Wilcoxon signed ranks test with Bonferroni's correction was used for the post-hoc analysis. To characterize total release of fHb, plasma NO-consumption, urinary NAG and plasma IFABP, the area under the curve (AUC_{fHb} , AUC_{NO} , AUC_{NAG} , and AUC_{IFABP}) was calculated for each patient using trapezoidal analysis with time as a baseline. Univariate correlations were Spearman correlations (R_s) and linear regression analysis (r^2). Multivariable linear regression analysis enabled correction for other confounding risk factors. Statistical calculations were made using SPSS 15.0 (SPSS, Inc., Chicago, IL), and Prism 4.03 (GraphPad Software Inc. San Diego, CA). Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Patient characteristics and outcome

Patients were predominantly male (N = 57, 85.1%) and middle-aged (mean age 66.5

years, range 38 - 82 years). Baseline demographic patient data did not differ significantly among patients undergoing OPCAB, CABG, or CABG+Valve surgery (**Supplemental Table S7.1**). Preoperative renal function was significantly lower in the latter two patient groups ($p = 0.003$). In total, 9 patients developed AKI (13.4%). Renal function recovered in all AKI patients prior to discharge and none required (temporary) dialysis. Serious gastro-intestinal complications such as bowel ischemia did not occur. Two patients (3.0%) developed a postoperative ileus which was successfully, non-surgically, treated. Overall in-hospital mortality was 1.5% ($N = 1$) and cause of death was respiratory failure with major neurological injury.

Supplemental table S7.1 Perioperative patient characteristics.

Characteristics	OPCAB (N=7)	CABG (N=30)	CABG + Valve (N=30)	p-value
Male	71.4 (5)*	90.0 (27)	83.3 (25)	0.367
Age (years)	64 (39 - 67)	71 (60 - 73)	72 (62 - 77)	0.054
BMI (kg/m^2)	25.0 (23.5 - 26.3)	27.7 (25.5 - 31.0)	26.3 (26.5 - 30.6)	0.138
Hypertension	57.1 (4)	90.0 (27)	83.3 (25)	0.067
Currently smoking	0.0 (0)	16.7 (5)	16.7 (5)	0.631
Hypercholesterolemia	57.1 (4)	86.7 (26)	86.7 (26)	0.074
Myocardial infarction	14.3 (1)	40.0 (12)	26.7 (8)	0.283
PAOD	28.6 (2)	16.7 (5)	10.0 (3)	0.078
COPD	0.0 (0)	16.7 (5)	13.3 (4)	0.596
Stroke	14.3 (1)	6.7 (2)	13.3 (4)	0.614
Ejection fraction <50%	28.6 (2)	30.0 (9)	43.3 (13)	0.116
Serum creatinine ($\mu\text{mol/L}$)	75 (69 - 78)	85 (78 - 91)	87 (77 - 97)	0.012
Estimated GFR (ml/min/1.73m^2)	88 (86 - 102)	76 (71 - 86)	71 (64 - 85)	0.003
Duration of surgery (min)	120 (108 - 179)	191 (158 - 240)	233 (188 - 263)	<0.001
ACC time (min)	-	40 (35 - 70)	106 (73 - 122)	<0.001
Duration of CPB (min)	-	68 (55 - 112)	145 (101 - 160)	<0.001
Transfused pRBCs (units)	0.0 (0)	1.5 (1.0 - 2.0)	2.0 (1.5 - 4.0)	<0.001
Use of pericardial suction	0.0 (0)	3.3 (1)	100.0 (30)	<0.001
Urine production (ml)	300 (92 - 720)	443 (264 - 593)	360 (273 - 493)	0.454
Blood loss during surgery (ml)	175 (150 - 300)	200 (120 - 300)	300 (190 - 530)	0.122
In hospital mortality	0 (0)	0 (0)	3.3 (1)	0.545
Increase of serum creatinine < 48h compared to preoperative value (%)	103 (94 - 109)	109 (96 - 118)	114 (101 - 125)	0.160
Acute kidney injury				0.603
Stage 0 (no AKI)	100.0 (7)	90.0 (27)	80.0 (24)	
Stage 1	0.0 (0)	10.0 (3)	16.7 (5)	
Stage 2	0.0 (0)	0.0 (0)	3.3 (1)	
Stage 3	0.0 (0)	0.0 (0)	0.0 (0)	
Duration of ICU admittance (days)	1.0 (1 - 1)	1.0 (1.0 - 1.0)	1.0 (1.0 - 2.0)	0.090
Duration of hospitalization (days)	6.0 (6.0 - 8.0)	7.0 (6.8 - 9.0)	7 (6.5 - 13.0)	0.148

*Values are % (N) or median (25th-75th IQR). Abbreviations: OPCAB, off-pump coronary artery bypass grafting; CABG, on-pump coronary artery bypass grafting; CABG+Valve, on-pump coronary artery bypass grafting and any type of valve reconstruction or replacement; PAOD, peripheral arterial occlusive disease; COPD, chronic obstructive pulmonary disease; GFR, glomerular filtration rate; ACC, aortic cross clamp; CPB, cardiopulmonary bypass; IC, intensive care unit.

CPB-assisted surgery causes acute hemolysis, increased plasma NO-consumption, and depletion of plasma haptoglobin

Baseline fHb levels were comparable between the three patient groups ($p = 0.78$, **Figure 7.1A**). During surgery, plasma fHb levels increased significantly in patients subjected to CPB ($p < 0.0001$ for CABG, and CABG+Valve surgery patients), peaking at the end of CPB ($6.5 \pm 0.9 \mu\text{mol/L}$, $p = 0.041$ compared to baseline for CABG patients, and $18.6 \pm 2.9 \mu\text{mol/L}$, $p < 0.0001$, for CABG+Valve patients). After cessation of CPB, plasma fHb concentrations gradually decreased over time in these patient groups. Both peak fHb levels and total release of fHb (AUC_{fHb}) was significantly correlated to CPB-duration ($R_s = 0.50$, $p < 0.001$, and $R_s = 0.55$, $p < 0.001$, respectively). Hemolysis did not occur in OPCAB patients (change of plasma fHb over time: $p = 0.22$). The kinetics of plasma NO-consumption was similar to that of plasma fHb in all study groups (**Figure 7.1B**). Plasma NO-consumption only significantly increased in patients subjected to on-pump surgery ($p < 0.001$ for CABG and CABG+Valve patients), while baseline NO-consumption was statistically equal between the study groups ($p = 0.18$). The NO-consuming capacity of plasma peaked at the end of CPB, having increased 2.4 fold in CABG patients, and 7.9 fold in CABG+Valve surgery patients. Consistent with the potent NO-scavenging property of fHb, the AUC_{fHb} correlated significantly to total plasma NO-consumption (AUC_{NO} , $r^2 = 0.75$, $p < 0.0001$, **Figure 7.1C**).

Physiologically, the plasma protein Hp acts as a buffer against increases in plasma fHb levels through the formation of Hp-fHb complexes resulting in fHb clearance from the circulation.¹⁷ This way, excessive hemolysis can potentially result in a significant reduction of Hp bioavailability.²⁰ Indeed, plasma Hp dropped significantly over time in CABG and CABG+Valve surgery patients ($p < 0.0001$, for both groups) suggestive of Hp-fHb complex clearance. Loss of circulating Hp was most marked in CABG+Valve surgery patients compared to CABG patients, which related to the higher plasma fHb levels in this patient group. The first statistically significant decrease in plasma Hp was measured at the end of CPB, and concentrations further declined during the postoperative period reaching lowest levels 2 hours and 4 hours after cessation of CPB in CABG and CABG+Valve surgery patients ($0.69 \pm 0.11\text{g/L}$, $p < 0.0001$, and $0.47 \pm 0.07\text{g/L}$, $p < 0.0001$, respectively). The maximum increase of plasma fHb correlated significantly to the maximum decrease of Hp ($r^2 = 0.28$, $p < 0.0001$, data not shown). Similar to fHb and NO-consumption, Hp levels remained relatively constant in OPCAB patients throughout surgery ($p = 0.059$). The observed increase of Hp after reaching lowest levels 4 hours after cessation of CPB was suggestive of enhanced Hp production and subsequent release of Hp into the circulation.

Figure 7.1 Acute hemolysis is associated with increased plasma NO-consumption and loss of Hp. Plasma levels of fHb (A), and NO-consumption (B) increased significantly during surgery in patients undergoing CABG+Valve (■), and CABG (●) surgery. Levels did not significantly change in OPCAB patients (▲). The total release of fHb and change in NO-consumption (C), estimated using the area under the curve (AUC) during surgery were significantly correlated ($r^2 = 0.75$, $p < 0.0001$). Figure D depicts the change in perioperative Hp levels in all groups. All values were corrected for hematocrit because of significant intraoperative hemodilution. Values are mean $\pm$ SEM (A, B, and D) or a scatter plot with mean (black line) $\pm$ 95% confidence interval (dotted line, C). Stars indicate significant changes compared to baseline within groups: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Numbers 1-8 refer to sample times of blood specimens (methods). CPB was not used in OPCAB patients.

CPB-induced hemolysis is associated with visceral injury and clinical acute kidney injury

The development of renal tubular injury and intestinal mucosal injury was studied using urinary NAG (**Figure 7.2A**) and plasma IFABP (**Figure 7.2B**). Both parameters were similar in all groups at baseline ($p = 0.859$, and $p = 0.697$, respectively), and increased significantly during CABG and CABG+Valve surgery ($p < 0.0001$, and $p < 0.001$ for NAG and IFABP, respectively). OPCAB patients did not display significant changes of NAG or IFABP over time ($p = 0.235$ and $p = 0.062$, respectively). The potential relation between acute hemolysis and visceral organ injury in patients undergoing cardiac surgery was

studied by correlation of AUC_{fHb} , AUC_{NAG} , and AUC_{IFABP} (**Figure 7.2C** and **Figure 7.2D**). Both renal injury and intestinal damage were significantly correlated with the extent of hemolysis during surgery ($R_s = 0.70$, $p < 0.0001$, and $R_s = 0.26$, $p = 0.04$, respectively), albeit this correlation was stronger for renal tubular injury. Importantly, the correlation between hemolysis, renal tubular damage and intestinal injury remained statistically significant after correction for four other potential confounders using multivariable logistic regression (**Table 7.1** and **Table 7.2**, respectively). The correlation between NO-consumption and both tissue injury markers was comparable to their correlation with fHb ($R_s = 0.55$, $p = 0.002$, and $R_s = 0.41$, $p = 0.03$, for NAG and IFABP respectively, data not shown), suggestive of a causal role of fHb-induced NO-scavenging in the development of visceral injury during cardiac surgery.

Figure 7.2 Acute hemolysis is significantly correlated to renal tubular injury and intestinal mucosal damage. Levels of urinary NAG (A) and plasma IFABP (B) changed significantly in patients undergoing CABG+Valve (■), and CABG (●) surgery, and in patients undergoing CABG+Valve, CABG, and OPCAB patients (▲), respectively. The total release (estimated using the area under the curve (AUC) of fHb and NAG (C), and fHb and IFABP (D) were significantly correlated ($R_s = 0.70$, $p < 0.0001$, and $R_s = 0.26$, $p = 0.04$, respectively). Plasma IFABP values were corrected for hematocrit because of significant intraoperative hemodilution, urinary NAG levels were corrected for urine creatinine values. Values are mean $\pm$ SEM (A, and B) or a scatter plot with mean (black line) $\pm$ 95% confidence interval (dotted line, C and D). Stars indicate significant changes compared to baseline within groups: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Numbers 1-8 in refer to sample times of blood specimens (methods). CPB was not used in OPCAB patients.

Table 7.1 Multivariable linear regression for AUC_{NAG}.

Variable	Beta	95% CI	p value
Age (years)	-0.04	-122.14 – 84.49	0.72
Preoperative eGFR (ml/min/1.73m ²)	0.01	-72.26 – 76.18	0.96
Duration of CPB (min)	-0.08	-21.82 – 10.19	0.47
Need for packed red blood cell transfusion (yes)	0.12	-1200.34 – 3530.49	0.33
AUC _{fHb}	0.77	0.70 – 1.32	<0.0001

Table 7.2 Multivariable linear regression for AUC_{IFABP}.

Variable	Beta	Confidence Interval (95%)	p value
Age (years)	0.16	-5763.55 - 191117.38	0.28
Cardiac Ejection Fraction (%)	0.24	-86399.79 - 544968.51	0.15
Intraoperative blood loss (mL)	-0.03	-401.87 - 339.21	0.87
Number of blood transfusions (units)	-0.15	-182970.49 - 78106.15	0.42
AUC _{fHb}	0.30	0.60 - 120.77	0.04

Finally, we studied the relation between hemolysis and development of AKI as defined by an increase in serum creatinine ≥ 150 % compared to preoperative values within 48 hours after surgery.¹⁵ Clinical characteristics of AKI and non-AKI patients are depicted in **Supplemental Table S7.2**. Patients developing postoperative AKI were already identifiable during surgery as they displayed significantly higher fHb levels at the end of CPB compared to non-AKI patients (24.4 ± 7.6 $\mu\text{mol/L}$ vs. 9.6 ± 1.2 $\mu\text{mol/L}$, $p < 0.01$, **Figure 7.3A**). AKI patients also showed significantly higher NAG levels at the end of CPB, indicating more profound renal tubular injury compared to non-AKI patients (11.9 ± 5.7 U/mmol vs 4.2 ± 0.5 U/mmol, $p < 0.05$, **Figure 7.3B**). We could not analyze NO-consumption in AKI and non-AKI patients due to small patient numbers as NO-consumption was only assessed in a (random) selection of patients.

Supplemental table 7.2 Clinical characteristics of AKI and non-AKI patients.

Characteristic	Non-AKI (N = 58)	AKI (N = 9)	p value
Type of surgery			0.176
OPCAB	12.1 (7)*	0.0 (0)	
CABG	46.6 (27)	33.3 (3)	
CABG+Valve	41.4 (24)	66.7 (6)	
Male	86.2 (50)	77.8 (7)	0.614
Age (years)	68 (57 - 73)	73 (66 - 77)	0.107
Ejection fraction <50%	23.1 (12)	33.3 (3)	0.119
Hypertension	81.0 (47)	100.0 (9)	0.334
PAOD	13.8 (8)	22.2 (2)	0.465
Serum creatinine ($\mu\text{mol/L}$)	83 (77 - 94)	83 (75 - 96)	0.797
Estimated GFR (ml/min/1.73m^2)	77 (69 - 86)	80 (62 - 92)	0.659
Duration of surgery (min)	195 (160 - 240)	245 (181 - 270)	0.147
ACC time (min)	69 (39 - 106)	78 (53 - 120)	0.255
Duration of CPB (min)	105 (63 - 148)	118 (79 - 159)	0.431
Transfusion requirement with stored blood	25.9 (15)	6 (66.7)	0.010
Use of pericardial suction	41.4 (24)	77.8 (7)	0.070
Urine production (ml)	388 (263 - 568)	415 (331 - 496)	0.800
Blood loss (ml)	245 (150 - 360)	200 (188 - 750)	0.542
In hospital mortality	1.7 (1)	0.0 (0)	1.000
Duration of ICU admittance (days)	1 (1 - 1)	1 (1 - 5)	0.111
Duration of hospitalization (days)	7 (6 - 8)	9 (8 - 14)	0.007

*Values are % (N) or median (25th-75th IQR). Abbreviations: OPCAB, off-pump coronary artery bypass grafting; CABG, on-pump coronary artery bypass grafting; CABG+Valve, on-pump coronary artery bypass grafting and any type of valve reconstruction or replacement; PAOD, peripheral arterial occlusive disease; GFR, glomerular filtration rate; ACC, aortic cross clamp; CPB, cardiopulmonary bypass; ICU, intensive care unit.

Figure 7.3 Patients with postoperative AKI display significantly more hemolysis and renal tubular injury during surgery. Patients with postoperative AKI (grey bars) display significantly higher fHb (A) and urinary NAG levels (B), compared to non-AKI patients (white bars). Values are mean + SEM. Stars indicate significant changes compared to baseline within groups: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Hash symbols indicate significant differences between groups: # = $p < 0.05$, ## = $p < 0.01$, ### = $p < 0.001$. Numbers 1-8 refer to sample times of blood specimens (methods). CPB was not used in OPCAB patients.

DISCUSSION

The current study demonstrates that hemolysis occurring during cardiothoracic surgery significantly increases plasma NO-consumption. Moreover, hemolysis is independently correlated to the development of visceral organ injury and AKI. Patients subjected to more complex types of cardiac surgery, such as combined bypass and cardiac valve surgery, appear to be at highest risk.

The adverse effect of hemolysis on the intravascular NO-metabolism is increasingly appreciated as an important pathophysiological mechanism in the development of complications in various clinical settings. The discovery of oxygenated plasma fHb ($\text{HbFe}^{2+}\text{O}_2$) as a potent scavenger of endothelial-derived NO (in a 1:1 stoichiometric and irreversible reaction) has provided an explanation for the development of gastrointestinal smooth muscle dystonias, vasculopathy, endothelial dysfunction, renal injury, pulmonary hypertension, and increased coagulation in patients with chronic hemolytic disease.^{10, 11} In the present study, acutely enhanced fHb concentrations during surgery caused a 2.4-fold increase in plasma NO-consuming capacity in CABG patients (compared to preoperative levels), and a 7.9-fold increase in CABG+Valve patients, in whom NO-consumption levels of 266 μmol per liter of plasma were observed. As plasma NO-consumption levels of approximately 10-15 $\mu\text{mol/L}$ already have been related to a significant impairment in NO-dependent fore-arm blood flow response in patients, the extent of NO-consumption measured in these patients is of clinical significance.^{10,12} Such a decrease in NO-bioavailability promotes vasoconstriction, platelet aggregation, upregulation of adhesion molecule expression, and stimulation of vascular leucocyte adhesion and inflammation.¹⁰

Hemolysis is a well-recognized, although considered relatively harmless, consequence of CPB, and has been shown to increase with longer perfusion times.^{9,21} Three factors principally contribute to the development of CPB-induced hemolysis: 1) exposure of RBCs to mechanical forces, particularly shear stress⁹, 2) contact of blood with air or non-endothelial surfaces²², and 3) use of suction pressures.²³ In addition, the development of sublethal RBC damage, which promotes premature intravascular RBC disintegration and worse, alters the rheological properties of blood contributing to increased fHb levels during surgery.²⁴ Besides CPB the use of autotransfusion devices and transfusion of stored red blood cells may contribute to increased fHb levels during cardiac surgery. Autotransfusion devices remove fHb from the salvaged blood, but cause significant sublethal damage to red blood cells.⁹ Retransfusion of such "less-viable" cells may increase plasma fHb levels after premature intravascular lysis. Prolonged storage of blood results in both hemolysis and sublethal damage of erythrocytes.²⁵ Therefore, transfusion of stored red blood cell products could cause a direct (transfusion of the fHb-containing storage medium), and delayed (disintegration

of less viable RBCs) increase of fHb in the patient (*Vermeulen Windsant et al, Crit Care 2012;15:R95*). In line, we measured highest fHb levels in CABG+Valve surgery patients who were exposed to the longest CPB times, and required the highest number of stored red blood cell units, while OPCAB patients did not display significant changes of plasma fHb over time.

As the development of hemolysis during CPB is well known, there have been several, albeit a few, studies reporting on the adverse relation between intraoperative hemolysis and patient outcome after cardiovascular surgery. These studies all focused on the direct toxic effect of fHb on the renal epithelium by fHb-induced generation of damaging reactive oxygen species (catalyzed by free heme and iron), and formation of obstructive casts.²⁶⁻³⁰ As fHb was believed to be merely nephrotoxic after glomerular filtration, a correlation between hemolysis and injury of other organs in the context of CPB has, to our knowledge, never been reported. The additional NO-scavenging property of fHb, next to its nephrotoxic effect, could however explain the stronger correlation between hemolysis and renal injury compared to the correlation between hemolysis and intestinal injury in this study. The fact that in this setting hemolysis correlates with injury of two independent organ systems has significant implications for the (potential) harmful effect of fHb on other tissues. It has been reported that fHb impairs left ventricular function and coronary blood flow in neonatal rabbit hearts, particularly following ischemia and reperfusion.³¹ Furthermore, the correlation between hemolysis and pulmonary hypertension in the setting of chronic hemolysis is well established.³²

The results of this study offer a window of opportunity to reduce surgery related morbidity and mortality, particularly in high risk patients. Inactivation of fHb or increasing NO-bioavailability may maintain vascular homeostasis, stimulate microcirculatory blood flow, and thus limit tissue injury during CPB-assisted cardiac surgery. Three therapeutic options are of particular interest in the current setting. First, prophylactic and/or therapeutical administration of (recombinant) Hp could counteract the depletion of Hp which we observed during and after surgery. In fact, administration of Hp significantly reduced urinary NAG levels and prevented hemoglobinuria in 14 patients undergoing CABG surgery with CPB.²⁷ Unfortunately, high costs currently limit clinical implementation of routine Hp supplementation, though plasma transfusion could be an option. Second, administration of NO-gas via the oxygenator or ventilation gas, analogous to inhaled NO (iNO), may prove to be beneficial. iNO, already at very low concentrations (80ppm), inactivates fHb in the pulmonary vasculature through oxidation, and reduces the NO-consuming capacity of plasma.¹⁰ iNO enhances the formation of NO-metabolites, such as nitrate and nitrite, which are important for intravascular NO transport, and cytoprotection.³³ Also, iNO decreases shear stress induced sublethal RBC damage.³⁴ Inhaled NO is already clinically

used in various settings³⁵, and it neither alters NO-consumption in healthy volunteers nor results in significant methemoglobinemia.¹⁰ Finally, the direct oral or intravenous administration of nitrite or nitrate to increase the plasma NO-donor pool could be worth investigating.³³

The main strength of our study is the inclusion of patients undergoing different types of cardiac surgery of various complexity. This allowed the identification of the group of patients at highest risk for hemolysis-induced organ injury who may benefit from hemolysis-targeted therapeutic interventions. Furthermore, the exclusion of patients with preoperative impaired renal function prevented confounding on the outcome measures renal tubular injury and postoperative AKI. The main limitations are inherent to the observational nature of the study. The strong and significant correlations between hemolysis, impaired NO-bioavailability, renal tubular injury, intestinal damage, and AKI should not be interpreted as causal relationships. Also, the number of adverse clinical events (AKI or significant intestinal complications) was relatively low, and thus precludes firm conclusions as to the impact of hemolysis on adverse clinical outcome. Finally, other potential mechanisms underlying the relation between intravascular hemolysis and kidney damage, such as heme induced oxidative damage were not investigated and may be important in this setting.³⁰

CONCLUSIONS

This study supports an adverse relation between enhanced plasma fHb levels, decreased plasma NO-bioavailability, and organ integrity loss during cardiac surgery. Furthermore, this study illustrates that patients with high intraoperative fHb levels are at higher risk for the development of postoperative AKI. The current results are of clinical importance as they aid in the identification of high risk patients who may benefit from therapeutic interventions aimed at the adverse effects of fHb. Preoperative plasma Hp could serve as a novel preoperative diagnostic and screening tool in the selection of patients with a low buffer capacity to increased fHb. Additionally, patients with impaired pre-operative renal function, undergoing complex cardiac surgery associated with long perfusion times and high transfusion requirements may be at particular high risk for the development of fHb-induced visceral tissue injury and AKI. These results call for increased awareness for the adverse consequences of even modest increases in plasma fHb during cardiac surgery. Finally, our study suggests that inactivation of fHb or supplementation of NO may significantly attenuate the development of visceral tissue injury and AKI, and improve patient outcome after CPB-assisted cardiac surgery.

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Blood transfusions increase circulating plasma
free hemoglobin levels and plasma nitric oxide
consumption: a prospective observational pilot study

Iris C Vermeulen Windsant, Norbert CJ de Wit, Jonas TC Sertorio, Erik AM Beckers,
Jose E Tanus-Santos, Michael J Jacobs, Wim A Buurman

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ABSTRACT

Introduction

The increasing number of reports on the relation between transfusion of stored red blood cells (RBC) and adverse patient outcome has sparked an intense debate on the benefits and risks of blood transfusions. Meanwhile, the pathophysiological mechanisms underlying this postulated relation remain unclear. The development of hemolysis during storage might contribute to this mechanism by release of free hemoglobin (fHb), a potent nitric oxide (NO) scavenger, which may impair vasodilation and microcirculatory perfusion after transfusion. The objective of this prospective observational pilot study was to establish whether RBC transfusion results in increased circulating fHb levels and plasma NO-consumption. In addition, the relation between increased fHb values and circulating haptoglobin, its natural scavenger, was studied.

Methods

Thirty patients electively received one ($N = 8$) or two ($N = 22$) stored packed red blood cell units. Blood samples were drawn to analyze plasma levels of fHb levels, haptoglobin, and NO-consumption prior to transfusion, and 15minutes, 30minutes, 60minutes, 120minutes, and 24hours after transfusion. Differences were compared using Pearson Chi-square or Fisher's exact test for dichotomous variables, or independent sample t-test or Mann-Whitney-U-test for continuous data. Continuous, multiple time-point, data were analyzed using the repeated one-Way ANOVA or Kruskal-Wallis test. Correlations were analyzed using the Spearman correlation.

Results

Storage duration correlated significantly with fHb concentrations and NO-consumption within the storage medium ($R = 0.48$, $p < 0.001$, and $R = 0.54$, $p < 0.01$). fHb also significantly correlated with NO-consumption directly ($R = 0.62$, $p = 0.002$). Transfusion of 2 red blood cell units significantly increased circulating fHb and NO-consumption in the recipient ($p < 0.001$ and $p < 0.05$, respectively), in contrast to transfusion of 1 stored red blood cell unit. Storage duration of the blood products did not correlate with changes in fHb and NO-consumption in the recipient. In contrast, pre-transfusion recipient plasma haptoglobin levels inversely influenced post-transfusion fHb concentrations.

Conclusions

These data suggest that RBC transfusion can significantly increase post-transfusion plasma fHb levels and plasma NO-consumption in the recipient. This may contribute to the potential pathophysiological mechanism underlying the much discussed adverse relation between blood transfusions and patient outcome. This observation may be of particular importance for patients with substantial transfusion requirements.

INTRODUCTION

Transfusion of stored red blood cells is a common medical procedure, particularly in the context of critical care, with approximately 70 million packed red blood cells (pRBC) being transfused worldwide each year.¹ The rationale of pRBC administration is enhancement of intravascular oxygen carrying capacity and thus improvement of tissue oxygenation. However, recent insights into pathological changes of RBC functionality and integrity during storage, collectively known as the “storage lesion”, has drawn attention to the potential negative consequences of pRBC transfusion as it limits the post-transfusion survival of RBCs *in vivo*.² The storage lesion includes decreased RBC viability and deformability, increased RBC aggregability and adhesiveness, enhanced susceptibility to oxidative damage, formation of membrane vesicles resulting in loss of surface area, and increased cell density.²⁻⁴ Furthermore, several changes to the RBC microenvironment (the storage medium) have been reported; a decrease in pH, accumulation of pro-inflammatory substances, hemoglobin-derived free iron, and microvesicles containing large amounts of free hemoglobin (fHb)⁵, and increased levels of hemolysis markers such as potassium, arginase -1, and fHb in the storage medium.^{2,6-8} Generally, increasing storage duration aggravates the storage lesion.²

The profound beneficial effects of transfusion cannot be understated. Nevertheless, the number of studies reporting on the potential adverse effects of (massive) pRBC transfusion on patient morbidity and mortality has increased significantly in recent years which has initiated an intense debate on the benefits and risks of pRBCs, particularly focusing on the effects of “younger” versus “older” pRBCs.⁹⁻¹⁴ Although the exact mechanisms underlying the adverse effects of pRBC administration have not yet been fully elucidated, the release of fHb and its influence on the intravascular nitric oxide (NO) metabolism after transfusion has been attributed an important role.⁵ FHB was found to be a potent scavenger of NO, the most important endogenous vasodilator.¹⁵ In line, increased fHb levels in patients with chronic and acute hemolysis have been associated with decreased NO-bioavailability within the microcapillary bed, decreased organ perfusion and increased organ injury.¹⁵⁻¹⁷ Similarly, transfusion of stored pRBC units may enhance fHb concentrations in patients after transfusion, for instance as a consequence of premature intravascular rupture of the transfused erythrocytes, or due to the transfusion of the fHb-containing storage medium.⁷ In line with this hypothesis, it was recently shown that transfusion of fHb-containing supernatant of stored erythrocytes in rats caused a significant increase in blood pressure which correlated with the fHb levels in the supernatant.⁵

This prospective observation pilot study aimed to investigate if transfusion of 1 or 2 pRBC units can result in enhanced circulating fHb levels and plasma NO-consumption

in the recipient. In addition, we studied whether pRBC storage duration or pre-transfusion recipient plasma haptoglobin (Hp) levels - the physiological fHb scavenger - influenced the change of plasma fHb in the recipient after transfusion.

MATERIALS AND METHODS

Patients

Based on previous experiments, we calculated that inclusion of thirty patients would provide sufficient power to detect significant increases in plasma fHb levels in the recipient after a blood transfusion. To that end, fifty-eight patients admitted to the Department of Hematology and Oncology of the Maastricht University Medical Center between May 1st 2010 and November 1st 2010 were screened for eligibility for study participation using predetermined inclusion and exclusion criteria. This resulted in the inclusion of thirty patients providing written informed consent at least 24 hours before transfusion. The study protocol was approved by the local ethical review board in accordance with the Declaration of Helsinki (amended in Seoul, 2008). We only included adult patients (≥ 18 years) with normal kidney function, and normal liver function, who were in a stable clinical phase of their disease. Normal kidney function was defined as an estimated glomerular filtration rate of 60 ml/min/1.73m² or higher, assessed by the abbreviated Modification of Diet in Renal Disease as recommended by the National Kidney Foundation.¹⁸ Normal liver function was indicated by normal pre-transfusion bilirubin levels. Furthermore, presence of a central venous line (in the jugular or subclavian vein) was mandatory to enable non-traumatic blood sampling, thus minimizing sampling-induced hemolysis. C-reactive protein levels exceeding 10 mg/L were reason for patient exclusion since an active inflammatory process may lead to increased Hp levels. As Hp is the physiological fHb scavenger, patients with increased Hp concentrations may be able to clear fHb more readily compared to patients with normal plasma Hp levels (0.25 – 1.9 g/L). As Hp is not routinely measured in patients prior to blood transfusion at our hospital we were not able to use Hp concentrations directly to include or exclude patients. Other exclusion criteria included pre-existent hemolytic disease, and transfusion of pRBCs within 72 hours prior to inclusion. We chose a time-frame of 72 hours on the basis of previous (unpublished) data showing that even after strong increases of plasma fHb, these levels are back to normal values at 24 hours after peak fHb levels were observed (in a stable clinical condition without ongoing hemolysis). The investigators did not have any influence on the selection of the transfused pRBC with regard to storage duration or any other parameter.

Red blood cell products

All transfused pRBCs were collected and prepared for transfusion by the Sanquin Blood Supply Foundation, the Netherlands. The maximum storage duration of pRBC units allowed in the Netherlands after the collection date is 35 days. All pRBC units were pre-storage leucodepleted, according to standard protocol. The storage solution consisted of a mixture of saline, adenine, glucose and mannitol. Mean volume and hematocrit of the transfused pRBC units were 275 mL and 0.60, respectively. The pRBCs were subsequently distributed to and stored by Central Diagnostic Laboratory of the Maastricht University Medical Center until use. In case 2 pRBC were administered, the storage duration of both pRBC units was never more than 2 days apart. The majority of transfused pRBCs were of blood type 0 (N = 32, 61.5%) and were Rh-negative (N = 33, 63.5%). The remainder of pRBC units were either of blood type A (N = 16, 30.8%) or B (N = 4, 7.7%). Eight patients were transfused with irradiated (25Gy) pRBCs. Each pRBC unit was administered in a 60-90 min time-frame. None of the patients received fresh frozen plasma or platelets during the study period.

Blood sampling and sample processing

Central venous blood was drawn from the venous line by an experienced nurse at 6 pre-set time points; pre-transfusion (blood sample taken on the morning of transfusion in the context of routine patient care), 15 minutes after transfusion (T15) of all pRBC (1 or 2), 30 minutes after transfusion (T30), 60 minutes after transfusion (T60), 120 minutes after transfusion (T120), and the morning after transfusion (T24). Prior to every sample collection, the central venous line was flushed with sterile saline to prevent sampling of the previously administered pRBC (which was given via the same line) or remnants of previously collected blood sample. Whole blood was gently collected using sterile syringes, instead of standard blood collection tubes (vacutainers), to prevent vacuum-induced hemolysis. After collection of two syringes, the blood from the second syringe (not contaminated with sterile saline) was immediately transferred to an EDTA-containing blood collection tube without vacuum (Becton Dickinson, Franklin Lakes, NJ), carefully mixed by gentle rotation, and subsequently stored for a maximum of 1 hour at 4°C, until further processing. After centrifugation (1500g at 4°C for 15 minutes without brake), plasma was aliquoted and stored at -20°C until further analysis.

Laboratory analysis

Free hemoglobin

FHb concentrations, indicating hemolysis, were measured in all patient plasma samples (N = 30) and in the storage medium of every administered pRBC (N = 52). FHb was

analyzed by derivative spectrometry as described in more detail elsewhere.¹⁹ The detection limit of this assay was 2 $\mu\text{mol/L}$.

Haptoglobin

Plasma Hp concentrations of all patient samples and Hp levels in the storage medium of all transfused pRBC were measured on a validated Beckman LX20 clinical chemistry analyzer (Beckman Coulter, Brea, CA) via a turbidimetric method by the Laboratory of Hematology of the Maastricht University Medical Center.

NO-consumption assay

To evaluate whether increased fHb levels influenced the NO-consuming capacity of plasma after transfusion, we analyzed NO-consumption in all plasma samples of 13 randomly selected patients from our total patient group of 30 (40%; 4 patients transfused with 1 pRBC, 9 patients transfused with 2 pRBC units), and in the storage medium of the 22 pRBCs administered to these patients. Random selection of patients was done using the statistical program SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL). Unfortunately, we were not able to analyze every sample from the total patient group due to time and cost constraints. The complete protocol of the NO-consumption assay is described in more detail elsewhere.^{15,20} In short, a 40 μM solution of the NO-donor, Diethylenetriamine NONOate (DETA NONOate, Cayman Chemical, Ann Harbor, MI) was prepared in PBS (pH 7.4) in a glass vessel purged with nitrogen in-line with a NO chemiluminescence analyzer (Sievers Model 280i, GE, Boulder, CO). The subsequent decay of DETA NONOate, releasing NO, produced a steady state NO signal of about 50–70 mV. When the signal became stable, 50 μL of plasma samples or standards were injected into the DETA NONOate solution, decreasing the NO signal in case of NO-consumption. Data were transferred to the software program ORIGIN Version 6.1 (OriginLab, Northampton, MA) for analysis of the area under the curve of decreasing NO-signal over time. The amount of NO consumption by plasma is quantified by comparison of the area under the curve with that of NO gas standards (produced from injections of nitrite into triiodide).

Statistical analysis

Continuous data in tables are presented as median and interquartile range (IQR, ranging the 25th–75th percentile), and as N (%) for dichotomous data. Continuous data presented in figures are depicted as mean $\pm$ standard error of the mean (S.E.M). Differences in patient characteristics between study groups were compared using Pearson Chi-square test for dichotomous variables, with Fisher's correction when appropriate, and the independent sample t-test or Mann–Whitney U-test for continuous data depending on Gaussian distribution (checked using histograms and normal Q–Q plots). Continuous data with multiple time-points were analyzed using the

repeated one-Way ANOVA with Bonferroni post-hoc correction, or the Kruskal-Wallis test with post-hoc Dunn's correction, depending on Gaussian distribution. Correlations were analyzed using the Spearman correlation (indicated as R_s). Statistical calculations were made using SPSS, and Prism 4.03 for Windows (GraphPad Software Inc. San Diego, CA). A p-value <0.05 was considered to indicate statistical significance.

RESULTS

Thirty patients were prospectively studied. Mean age of the total patient group was 52.4 years and the majority of patients were male (N = 25, 83.3%). Most patients had been diagnosed with acute myeloid leukemia (N = 21, 70%) and all patients were in a stable clinical phase of their disease. Eight patients (26.7%) were transfused with 1 pRBC, and 22 patients (73.3%) with 2 pRBC units. Interestingly, the increase in Hb following transfusion was similar in patients receiving 1 or 2 pRBC units: 8.2 g/L to 9.7 g/L. Also, other baseline and pRBC unit characteristics did not statistically differ between the two groups (Table 8.1).

Prolonged storage of pRBC results in elevated fHb levels and increased NO-consuming capacity of the storage medium

In total, 52 pRBCs were administered. The mean storage duration of the pRBC units was 17.2 days (ranging 2 - 32 days, Figure 8.1A), which was similar to previous reports.¹¹ Of all transfused units, 4 pRBC (7.2%) were younger than 1 week while 11 units (21.2%) had been stored for 3 weeks or longer. To analyze the degree of hemolysis within the pRBC during storage, we measured fHb concentrations in the storage medium of each transfused unit. The mean fHb level of the storage medium was 25.9 ± 2.2 $\mu\text{mol/L}$, with a peak level of 96.7 $\mu\text{mol/L}$. Corresponding to previous reports^{5,7}, prolonged storage duration correlated with fHb concentrations within the storage medium ($R_s = 0.48$, $p < 0.001$, Figure 8.1B). As fHb is a potent NO-scavenger, we hypothesized that fHb concentrations within the storage medium would positively correlate with the level of NO-consumption of the storage medium. NO-consumption of the storage medium averaged 76.0 $\mu\text{mol/L}$ (ranging 8.6 – 175.1 $\mu\text{mol/L}$), and was significantly correlated with fHb levels of the storage medium ($R_s = 0.62$, $p = 0.002$, Figure 8.1C), and storage duration of the pRBC ($R_s = 0.54$, $p = 0.01$, data not shown).

Table 8.1 Patient and packed red blood cell unit characteristics.

	Patients receiving 1 pRBC (N = 8)	Patients receiving 2 pRBC (N = 22)	p-value
<i>Patient characteristics</i>			
Age (years), median (IQR 25%-75%)	51.0 (23.0-61.0)	58.5 (43.5-68.0)	0.118
Male, % (N)	87.5 (7)	81.8 (18)	1.000
Disease % (N)			0.530
Acute myeloid leukemia	75.0 (6)	68.2 (15)	
Multiple myeloma	12.5 (1)	-	
Burkitt's lymphoma	-	4.5 (1)	
Acute lymphoblastic leukemia	-	4.5 (1)	
Testis carcinoma	12.5 (1)	-	
Myeloid sarcoma	-	4.5 (1)	
Non-Hodgkin B-cell lymphoma	-	9.1 (2)	
Myelodysplastic lymphoma	-	9.1 (2)	
Pre-transfusion Hb (g/dL), median (IQR 25%-75%)	8.2 (7.8-8.5)	8.2 (7.5-8.4)	0.383
Post-transfusion Hb (g/dL), median (IQR 25%-75%)	9.7 (9.0-10.0)	9.7 (9.2-10.2)	0.459
Pre-transfusion plasma haptoglobin (g/L), median (IQR 25%-75%)	2.1 (1.3-3.8)	2.4 (1.5-2.8)	0.784
Pre-transfusion serum bilirubin (μmol/L), median (IQR 25%-75%)	16.8 (12.2-28.0)	15.0 (13.4-22.3)	0.872
Pre-transfusion serum AST (IU/L), median (IQR 25%-75%)	16.0 (14.0-44.0)	14.0 (11.0-21.0)	0.231
Pre-transfusion serum ALT (IU/L), median (IQR 25%-75%)	28.0 (17.0-50.0)	24.5 (14.8-35.3)	0.507
Pre-transfusion serum creatinine (mmol/L), median (IQR 25%-75%)	65.0 (57.0-75.0)	67.0 (58.3-85.5)	0.508
<i>pRBC characteristics</i>			
Blood type, % (N)			1.000
O	75.0 (6)	59.1 (13)	
A	25.0 (2)	31.8 (7)	
B	-	9.1 (2)	
AB	-	-	
Rh negative, % (N)	62.5 (5)	59.1 (13)	1.000

Abbreviations: IQR, interquartile range; GFR, estimated glomerular filtration rate, calculated according to the MDRD; Hb, hemoglobin; AST, aspartate transaminase; ALT, alanine transaminase; pRBC, packed red blood cell unit. There were no significant changes between the study groups for any of the variables shown.

Figure 8.1 Storage duration of packed RBCs is correlated with increased fHb-concentrations and NO-consumption within storage medium. (A) Histogram of storage duration of all transfused pRBC units (N = 52). Mean storage duration was 17.2 days (ranging 2 - 32 days). (B) Correlation between storage duration and the fHb concentrations of the storage medium of each pRBC unit (N = 52). The black line depicts a non-linear regression (exponential) curve. (C) Correlation between the fHb concentration and NO-consumption within the storage medium in a random selection 22 pRBC units. The black line depicts a non-linear regression (exponential) curve. Abbreviations: pRBC, packed red blood cells; fHb, free hemoglobin; NO, nitric oxide; SM, storage medium.

Transfusion of 2 pRBCs results in increased plasma fHb concentrations and NO-consumption in patients

Next, we studied the effect of pRBC transfusion on plasma fHb levels of the 30 recipients. **Figure 8.2A** depicts the baseline and post-transfusion fHb concentrations, grouped according to transfusion of 1 or 2 pRBC units. This subdivision was made based on the notion that the latter group would (theoretically) be subjected to a higher fHb load and could thus display higher plasma fHb values after transfusion. Baseline plasma fHb levels were statistically similar between both groups. Interestingly, plasma fHb values increased significantly in patients receiving 2 pRBCs compared to baseline concentrations as early as 15 minutes after transfusion (from $2.6 \pm 0.2 \mu\text{mol/L}$ to $4.6 \pm 0.5 \mu\text{mol/L}$, respectively, $p < 0.001$), and fHb levels remained significantly enhanced compared to baseline values throughout the next two hours. After 24 hours, fHb levels had returned to pre-transfusion levels. Plasma fHb concentrations in patients transfused with 1 pRBC unit did not change significantly over time. Both storage duration and fHb concentrations of the storage medium of the administered pRBCs were similar between patients transfused with 1 or 2 pRBC units ($p = 0.63$ and $p = 0.97$, respectively), and it is therefore less likely that these factors confound the observed differences in plasma fHb concentrations after transfusion between both groups. It has been reported that irradiation of stored pRBC increases the storage lesion, especially membrane permeability.⁶ To exclude the potential confounding effect of pRBC irradiation on posttransfusion fHb concentrations in patients, we compared fHb concentrations in the storage medium of irradiated ($N = 13$) and non-irradiated pRBC units ($N = 39$); which were $23.6 \pm 2.7 \mu\text{mol/L}$ and $26.7 \pm 2.7 \mu\text{mol/L}$, respectively ($p = 0.97$). Moreover, posttransfusional fHb concentrations in plasma were not significantly different between patients receiving irradiated blood products versus patients receiving non-irradiated blood products ($p > 0.05$ on all time-points, data not shown).

Subsequently, NO-consumption was measured in all plasma samples of a randomly selected subgroup of 13 patients (**Figure 8.2B**). In line with the fHb-data, plasma NO-consumption rose significantly in patients receiving 2 pRBCs from $6.1 \pm 1.5 \mu\text{mol/L}$ at baseline to $7.9 \pm 1.3 \mu\text{mol/L}$ 15 minutes after transfusion ($p = 0.03$), remaining elevated during the first hour after transfusion ($p = 0.06$). Plasma NO-consumption did not significantly change in patients receiving 1 pRBC unit. Overall, NO-consumption in plasma correlated significantly with plasma fHb concentrations ($R_s = 0.35$, $p = 0.003$, data not shown).

Summarizing, the storage medium of stored pRBC units is a potent NO-consuming medium as it contains high levels of fHb. In line, plasma levels of fHb and NO-consumption increased significantly in patients after transfusion of 2 pRBC units.

Figure 8.2 Transfusion of (multiple) stored RBC units significantly increases plasma fHb and NO-consumption in patients. (A) Course of plasma fHb in patients receiving 1 (white bars) or 2 (grey bars) pRBC units from baseline levels (before blood transfusion) to 15 minutes, 30 minutes, 60 minutes, 120 minutes, and 24 hours after blood transfusion. Stars indicate significant changes of fHb levels within groups compared to baseline fHb concentrations; ***: $p < 0.001$). (B) Course of plasma NO-consumption in a random selection of 4 patients transfused with 1 pRBC, and 9 patients transfused with 2 pRBC. Stars indicate significant differences of fHb levels within groups compared to baseline fHb concentrations; *: $p < 0.05$). Abbreviations: BT, blood transfusion; pRBC, packed red blood cells; fHb, free hemoglobin; NS, non-significant; min, minutes.

Pre-transfusion haptoglobin levels influences post-transfusion plasma fHb concentrations in patients

The current debate concerning the assumed negative effect of transfusion of pRBC units on patient outcome mainly focuses on storage-related factors such as storage duration. As we found a significant effect of storage duration on fHb levels and NO-consumption within the storage medium, we studied whether storage duration of the pRBC units influenced post-transfusion fHb concentrations in vivo. Surprisingly, in our study, the storage duration of the pRBC unit did not correlate with post-transfusion fHb concentrations or NO-consumption in the recipient, even after correction for the number of pRBCs transfused, and fHb concentrations of the storage medium (data not shown). Next, we studied the effect of pRBC transfusion on post-transfusion Hp concentrations in the recipient. As Hp is the physiological scavenger protein for fHb, and considering the significant increase of circulating fHb concentrations following transfusion of 2 pRBC units, we expected decreased post-transfusion Hp levels in these patients. However, Hp levels did not change significantly after transfusion in patients receiving 1 pRBC or 2 pRBC units (**Figure 8.3A**). We subsequently investigated whether pre-transfusion Hp concentrations of the recipient influenced post-transfusion fHb levels, as patients with low pre-transfusion haptoglobin levels may have a lower capacity to clear fHb. To that end, we divided patients into three groups based on their pre-transfusion plasma haptoglobin level. Eighteen patients (60%) displayed high pre-transfusion haptoglobin concentrations exceeding 1.9 g/L (upper normal level). In contrast, 3 patients (10%) had haptoglobin concentrations below 0.75 g/L. Post-transfusion fHb concentrations showed a more pronounced increase from pre-transfusion levels in patients with lowest pre-transfusion Hp concentrations, compared to patients with higher pre-transfusion haptoglobin levels ($p < 0.05$, **Figure 8.3B**). Storage duration of the administered pRBCs was similar between the three groups ($p = 0.20$), as was the mean number of pRBC transfused (1 or 2, $p = 0.85$), and mean fHb concentration of the storage medium of the pRBC units ($p = 0.53$). Furthermore, similar results were obtained when patients receiving 1 or 2 pRBC units were analyzed separately (data not shown). Unfortunately, the study was not sufficiently powered to allow statistical analysis of changes in post-transfusion plasma NO-consumption based on pre-transfusion Hp levels.

DISCUSSION

The relation between transfusion of stored blood and adverse outcome in patients is subject of intense debate. Although the development of functional and structural changes of the RBC during storage is undisputed, it remains controversial whether these changes carry any clinical relevance. In recent years, several studies reported on the association between increased storage duration of pRBCs (reflecting increased storage lesion) and adverse outcome in trauma patients²¹, septic patients^{12,22} the critically ill²³, and patients undergoing cardiac surgery.^{11,24} In contrast, other investigators did not find such associations.^{13,14,25,26} The development of hemolysis within the pRBC unit, with release of the NO-scavenging protein fHb, may contribute to the potential adverse relation between blood transfusions and patient outcome.⁵

This observational pilot study investigated if 1) transfusion of 1 or 2 stored pRBC units can result in increased fHb concentrations and plasma NO-consumption in the recipient, and 2) whether the increase in fHb levels is dependent on storage duration of the pRBC unit(s) or on pre-transfusion recipient Hp levels. First, we demonstrated that prolonged storage significantly increased fHb concentrations and NO-consumption within the pRBC storage medium, which was in line with previous reports.^{7,27} Second, we were able to demonstrate for the first time that transfusion of 2 pRBCs significantly enhanced plasma fHb concentrations and NO-consumption in patients. Third, the increase in post-transfusion fHb concentrations was most profound in patients with low pre-transfusion Hp levels and was absent in patients with highest Hp concentrations, irrespective of the number of transfused pRBCs, storage duration of the transfused units, and fHb concentrations within the storage medium.

To date, studies reporting on the postulated adverse effects of pRBC transfusion on patient outcome principally focused on the contribution of pRBC-related factors, with most attention being paid to the effect of storage duration. We could not confirm a correlation between prolonged storage and increased fHb levels (or increased NO-consumption) in our patients. It could be that the uneven distribution of the storage duration of the pRBC units and the relatively small number of patients prevented us from finding such a relation. Interestingly however, we were able to show an effect of pre-transfusion plasma Hp levels of the recipient on changes of fHb following transfusion. High plasma Hp levels prior to transfusion may improve the physiological buffer capacity of the recipient to increased plasma fHb concentrations. It could even be hypothesized that patients displaying increased Hp levels, such as patients with profound systemic inflammation^{28,29}, may be better protected against the potential adverse effects of transfused fHb. In contrast, patients undergoing (complex) cardiovascular surgery could be particularly sensitive to transfusion-induced, fHb-

mediated, NO-consumption, as these patients frequently suffer Hp-depletion during surgery.³⁰

Our data may be of significant importance for patients who require massive transfusion, such as critical care patients, trauma patients or patients undergoing major aortic, cardiac, orthopedic, or gynecologic surgery.^{17,31} Transfusion of multiple pRBC units may result in a significant increase of plasma fHb levels and NO-consumption. Since plasma fHb concentrations of 6 $\mu\text{mol/L}$ have been shown to significantly impair the NO-metabolism in vivo¹⁵, and levels over 10 $\mu\text{mol/L}$ have been linked to the development of renal injury and renal dysfunction¹⁷, excessive pRBC transfusion may potentially decrease microcirculatory blood flow and contribute to organ compromise in these patients. Indeed, it has been shown that transfusion of pRBCs to treat anemia during CPB was independently associated with increased urinary markers of intestinal damage, renal injury, and deterioration of kidney function compared to untreated anemic patients.^{32,33} A similar independent association between pRBC transfusions and worse outcome was found in anemic critically ill patients.³⁴

Although the presented associations between the number of transfused pRBC units and increased levels of fHb and NO-consumption do not imply causality, the results of the current study elicit an interesting dilemma regarding blood transfusions. On the one hand, refraining from transfusing a (seriously) anemic patient might further impair blood oxygen carrying capacity and tissue oxygenation, possibly causing tissue ischemia and organ failure. On the other hand, (massive) transfusion of pRBC units, containing high amounts of fHb, could significantly increase plasma fHb concentrations and NO-consumption in the recipient. This could hamper microcirculatory blood flow and may ultimately lead to cellular damage and organ failure as well. As the beneficial effects of blood transfusions are undisputed, and safe alternatives to blood storage are still lacking, efforts should be made to minimize the unwanted effects of stored red blood cell transfusion. If our results are confirmed by larger studies, the current study provides opportunities for the development of preventive or treatment strategies.

Although we were able to demonstrate significant results, the relatively small patient population prevented correction for the confounding variables such as disease severity. Therefore, future studies are essential to provide additional insight into the (causal) relationships between transfusion of stored blood, increased plasma fHb concentrations and plasma NO-consumption, decreased microcirculatory perfusion, and clinical outcome of the recipient. Furthermore, we did not study the contribution of RBC microvesicles, known to be released during hemolysis and known to contain high concentrations of fHb, on post-transfusional fHb concentrations in the recipient.³⁵ In addition, the role of increased free (non-transferrin-bound) iron levels was not studied³⁶. Notwithstanding, we consider that the results of this pilot study provides

important additional insight into the much discussed relationship between stored blood transfusion and adverse outcome in patients, and warrants additional investigation.

CONCLUSIONS

Our data indicate that transfusion of 2 units of stored red blood cells significantly increases plasma fHb and NO-consumption in the recipient. These data are of interest in light of the ongoing debate and evaluation of the proposed negative association between pRBC transfusions and patient outcome, and may serve as a tool to improve patient morbidity and mortality after transfusion.

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Hemolysis-induced organ damage is reduced by nutritional activation of the vagal anti-inflammatory reflex

Jacco-Juri de Haan, Iris C Vermeulen Windsant, Tim Lubbers, Sebastiaan J Hanssen,
Mohammed Hadfoune, Frits W Prinzen, Jan Willem M Greve, Wim A Buurman

Submitted

A grayscale microscopic image showing several red blood cells. The cells are biconcave discs, with some in sharp focus in the foreground and others blurred in the background. A large, white, stylized number '9' is overlaid on the right side of the image.

9

ABSTRACT

Background

Acute hemolysis is a common clinical event associated with organ damage and inflammation and forms as such a target for intervention. Recently, stimulation of the cholecystokinin-1 receptor (CCK-1R)-dependent vagal anti-inflammatory reflex with lipid-rich enteral nutrition was demonstrated to preserve organ integrity and attenuate inflammation.

Objective

To investigate the effects of nutritional activation of the vagal anti-inflammatory reflex on tissue integrity and inflammation following acute hemolysis.

Methods

Intravascular hemolysis in male Sprague-Dawley rats was simulated by continuous infusion of cell-free hemoglobin (fHb). Animals were fasted or received lipid-rich enteral nutrition prior to fHb administration. CCK-1R agonist pegylated (PEG)-CCK9, CCK-1R antagonist A70104 and nicotinic acetylcholine receptor (nAChR) antagonist chlorisondamine were applied to investigate involvement of the vagal anti-inflammatory reflex. Blood and tissue were harvested at 2 hours post-fHb to assess organ integrity and inflammatory status. Microcirculatory changes at organ level were evaluated using fluorescent microspheres.

Results

Induction of clinically relevant plasma fHb concentrations resulted in organ compromise and systemic inflammation. Nutritional intervention attenuated renal tubular cell damage ($p < 0.05$), hepatocyte damage ($p < 0.05$), ileal leakage of horseradish peroxidase ($p < 0.01$) and bacterial translocation ($p < 0.05$) compared with fasted controls. Also circulatory IL-6 levels were decreased by enteral nutrition ($p < 0.05$). A70104 as well as chlorisondamine reversed the protective nutritional effects ($p < 0.05$), whereas PEG-CCK9 mimicked the impact of enteral feeding in fasted animals ($p < 0.05$). Moreover, nutritional intervention promoted renal, hepatic and intestinal perfusion ($p < 0.05$).

Conclusions

Lipid-rich nutrition preserves organ integrity and attenuates inflammation following fHb administration. These protective effects are CCK-1R- and nAChR-dependent, indicating a crucial role for the vagal anti-inflammatory reflex. These findings define intervention with enriched enteral nutrition as a potential therapy in patients prone to develop hemolysis.

INTRODUCTION

Intravascular hemolysis occurs in diverse critical care settings, e.g. in patients undergoing extracorporeal circulation or membrane oxygenation or in patients with a sickle cell crisis or autoimmune hemolytic anemia. Increased circulatory levels of cell-free hemoglobin (fHb), as observed during hemolysis, have been associated with the development of endothelial dysfunction and organ injury.¹⁻⁴ Crucial in the toxic effects of oxygenated fHb is its ability to irreversibly react with nitric oxide (NO), leading to the formation of methemoglobin (metHb) and nitrate.^{5,6} NO is a pivotal signal molecule in the regulation of smooth muscle relaxation, endothelial adhesion molecule expression, and platelet activation and aggregation. Massive NO scavenging leads to an impaired vascular function, decreased organ perfusion and eventually to tissue damage.⁷⁻⁹ In addition, elevated fHb levels limit the formation of NO carrier S-nitrosohemoglobin and decrease the availability of L-arginine, the principal NO donor.¹⁰⁻¹² The deficit of NO, which functions as a potent antiinflammatory mediator, as well as the presence of organ damage as a result of compromised organ perfusion, may contribute to the pro-inflammatory state observed in hemolytic disease. Therefore, therapies are needed to counteract the detrimental consequences of hemolysis.

Nutritional activation of the vagal anti-inflammatory reflex is a novel approach to reduce organ damage and counteract excessive inflammation.^{13,14} Lipid-rich enteral nutrition activates the autonomic nervous system via activation of cholecystokinin-1 receptors (CCK-1R) on afferent vagal fibers.¹⁴ In turn, parasympathetic outflow suppresses cytokine release via binding of cholinergic neurotransmitters to nicotinic acetylcholine receptors (nAChR) on inflammatory cells.^{13,15,16} Compelling evidence of improved outcome by nutritional, pharmacological and electrical stimulation of this neuroimmune axis, designated the cholinergic anti-inflammatory pathway, has been provided in diverse experimental models of acute and chronic inflammation.¹⁷⁻¹⁹

In addition to its neural and inflammatory properties, acetylcholine is recognised as a potent vasodilator acting largely independent of NO.²⁰⁻²² Since the sensibility for NO-independent vasoactive mechanisms is increased in hemolytic disease, we hypothesize that nutritional vagus stimulation resulting in acetylcholine release counteracts the vasoconstrictive effects of NO depletion, thus preventing the development of organ compromise.^{23,24}

The current study investigates the effects of lipid-rich enteral nutrition on renal, hepatic and intestinal organ perfusion and organ integrity in a setting of acute hemolysis. Also the nutritional effects on hemolysis-induced inflammation are assessed. Involvement of the vagal anti-inflammatory reflex is determined using CCK-1R agonists and antagonists and nAChR antagonists. The clinical scenario of ongoing hemolysis is mimicked in rodents by continuous infusion of fHb and other erythrocyte

constituents. Together, using a physiological and straightforward nutritional approach, this study may contribute to the development of novel means to decrease hemolysis-related complications in critical care patients.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 380 - 430 g, were purchased from Charles River Laboratories (Maastricht, the Netherlands) and housed under controlled conditions of temperature and humidity. Prior to the experiments, rats were fed standard rodent chow ad libitum and had free access to water. The experimental protocols were approved by the Animal Ethics Committee of the Maastricht University Medical Centre+.

Acute hemolysis model

The hemolysis model in rodents was based on a canine model described by Minneci and colleagues.¹ After induction with 4% isoflurane, anesthesia was maintained at 2% throughout the procedure. Acute hemolysis was simulated by administration of a 300 μ M fHb bolus of 0.65 mL / 100 g body weight followed by continuous intravenous infusion of 300 μ M fHb for 1 hour (1.3 mL / 100 g body weight). The left femoral artery was dissected and cannulated with polyethylene tubing (PE-10) containing heparinized saline (10 IU/mL). The cannule was connected to a computer-assisted external pressure transducer to register the mean arterial pressure (Kent Scientific, Torrington, CT). Animals were sacrificed at 2 hours after start of fHb administration (**Figure 9.1**).

Figure 9.1 Experimental design. Rats were food deprived (FD) 18 hours before start of free hemoglobin (fHb) infusion or received lipid-rich enteral nutrition at -18 hours, -2 hours and -45 mins. At 30 mins before fHb infusion, A70104, PEG-CCK9, chlorisondamine or vehicles were administered. Animals received fHb infusion for 1 hour and were sacrificed at +2 hours (†). Microspheres (M) were administered at four time points.

Generation and measurement of fHb

To generate fHb for infusion, fresh blood samples were obtained from healthy male Sprague Dawley rats through aortic puncture. After diluting whole blood 1:2 in saline containing 10 IU/mL heparin, erythrocytes were isolated by centrifugation (2,750 g, 15 minutes, 4°C). Plasma and buffy coat were carefully discarded and the remaining erythrocytes were washed three times in sterile saline (1:3 v/v) to remove all plasma components. To induce hemolysis, the erythrocytes underwent three freeze-thaw cycles after the final washing step. Erythrocyte membranes were removed by ultracentrifugation (20,000 g, 30 mins, 4°C). Next, the supernatant was passed through a micropore filter (diameter 0.22 µm, Millipore, Billerica, MA). The final concentration was adjusted with sterile saline to 300 µM for infusion. Routine spectrophotometry (Department of Clinical Chemistry) was used to assess the percentages of oxygenated hemoglobin and methemoglobin of total fHb. The fHb concentrations in infusate and plasma were measured by derivative spectrophotometry.²⁵

To investigate the role of NO scavenging by fHb in the development of organ damage in our hemolysis model, we hypothesized that exposure of fHb to NO before infusion, resulting in the formation of methemoglobin, would subsequently reduce the ability of fHb to scavenge NO in vivo. Therefore, a batch of fHb was pre-saturated with NO gas (Linde Gas, Dieren, the Netherlands) using a hollow fiber oxygenator (CAPIOX RX, Terumo, Somerset, NJ). The oxygenation level before and after exposure to NO gas was measured using routine spectrophotometry.

Interventions

Before fHb infusion, rats were fasted overnight (18 hours) or treated with liquid nutrition enriched with phospholipids given per oral gavage at three time points as described previously (**Figure 9.1**).¹³ The feeding contained 50.4 energy percent (en%) lipid, 8.7en% protein, and 40.9en% carbohydrates. The fat fraction contained 30% phospholipids. The lipid source was lecithin. Omega 3 and omega 6 fatty acids constituted less than 5 wt% (weight percent, < 5 g/100 mL). Proteins were derived from lean milk powder, containing 80% casein and 20% whey protein. The carbohydrate source was a mixture of sucrose and maltodextrins (Glucidex 19DE). The total nutrition provided constituted 10.0 en% of daily intake.

To investigate the role of peripheral CCK-1R in the nutritional effects on hemolytic damage, fed rats received A70104, also known as A65186 (100 µg/kg kindly provided by Abbott Laboratories, Abbott Park, IL), a CCK-1R antagonist that does not cross the blood-brain barrier.²⁶ A70104 was dissolved in sterile saline with 1% dimethyl sulfoxide. The role of CCK-1R was further assessed in fasted animals by administration of 6 µg/kg PEG-CCK9 (kindly provided by Dr Verbaeys, Interdisciplinary Research

Center, Campus Kortrijk, Katholieke Universiteit Leuven, Kortrijk, Belgium) dissolved in sterile saline. PEG-CCK9 specifically binds to CCK-1R and does not cross the blood brain barrier.²⁷ Involvement of peripherally localized nAChR was studied by administration of nAChR antagonist chlorisondamine diiodide (125 µg/kg; Sigma, St. Louis, MO), dissolved in saline. Antagonists and agonists were administered intravenously at 30 minutes before fHB infusion (**Figure 9.1**).

Parameters of organ integrity

To assess renal tubular damage, urinary N-acetyl-β-D-glucosaminidase (NAG) was measured by an enzyme colorimetric assay according to the manufacturer's instructions (HaemoScan, Groningen, the Netherlands). NAG, excreted predominantly by proximal renal tubular cells, is a relatively large molecule (130 kDa), precluding glomerular filtration. Furthermore, NAG is not reabsorbed by the tubules. Therefore, high levels of urinary NAG indicate renal tubular damage.²⁸ Results were normalized to urinary creatinine values and expressed as Units/mmol creatinine.

Liver fatty acid binding protein (L-FABP) concentrations in plasma were assessed as a marker of liver damage. L-FABP was determined using a standard ELISA for rat L-FABP (Hycult Biotech, Uden, the Netherlands). L-FABP is an abundant cytosolic protein in hepatocytes. Lower concentrations are expressed in the intestine, kidney, lung and stomach.^{29,30}

Intestinal permeability for macromolecules was assessed by measuring translocation of the 44 kD enzyme horseradish peroxidase (HRP) through the ileal wall.³¹ Segments of distal ileum (8 cm) were washed, everted, filled with 1 ml of Tris buffer (125 mmol/L NaCl, 10 mmol/L fructose, 30 mmol/L Tris; pH 7.5) and ligated at both ends. The filled segments were incubated in Tris buffer containing 40 µg/mL of HRP (Sigma). After incubation at room temperature for 45 minutes, ileal content was carefully collected. HRP activity was measured spectrophotometrically at 405 nm after addition of tetramethylbenzidine as a substrate.

Bacterial translocation to mesenteric lymph nodes, liver and spleen was determined as described previously.³²

Systemic inflammation

Systemic inflammation was assessed by detection of interleukin-6 (IL-6) concentrations in arterial blood using a standard ELISA for rat IL-6 (R&D Systems, Minneapolis, MN).

Microcirculatory blood flow

Assessment of microcirculatory blood flow was performed using fluorescent microspheres (diameter 15 µm) as described previously.^{33,34} Fluorescent microspheres (2.5×10^5 , Dye-Trak, Triton Technology, San Diego, CA) were infused at 10 minutes

before fHb (lemon) and at 30 minutes (persimmon), 60 minutes (orange) and 120 minutes (yellow) after start of fHb infusion via a PE-10 cannula inserted in the right femoral artery that was transferred into the aortic arch (**Figure 9.1**). A reference blood sample from the femoral artery was taken at every time point. After sacrifice, 1.5 - 2 gram of kidney, liver and small intestine was harvested for microsphere retrieval. Following organ lysis in potassium hydroxide, the fluorescent dye was dissolved in diethylene glycol monoethyl ether acetate (Sigma). Fluorescence was measured using an automated fluorospectrometer (PerkinElmer LS 50B, Perkin Elmer, Waltham, MA) and the flow to each individual organ was calculated. The flow before fHb infusion was set at 100% and flow changes at organ level during the experiments were determined for each individual subject.

Statistical analysis

All groups consisted of 6 animals. For between-group comparisons, a two-tailed Mann-Whitney U test was used. Differences were considered statistically significant at $p < 0.05$. Data are displayed as mean $\pm$ SEM. Prism 5.02 for Windows (GraphPad Software Inc., San Diego, CA) was used for computations.

RESULTS

Cell-free hemoglobin results in loss of organ integrity via impairment of nitric oxide bioavailability

Continuous administration of fHb resulted in strongly enhanced plasma fHb levels at 30 minutes ($40 \pm 3 \mu\text{M}$) that remained stable until termination of fHb infusion at 1 hour ($43 \pm 4 \mu\text{M}$). At 120 minutes, fHb concentrations dropped to $16 \pm 1 \mu\text{M}$, indicating that the half time of fHb in circulation is approximately 40 minutes. Next, we investigated whether the observed circulatory fHb levels compromised organ integrity. Infusion of fHb resulted in renal tubular cell damage, as indicated by strongly increased urinary NAG levels compared to sham controls ($p < 0.01$; **Figure 9.2A**). Next, enhanced plasma levels of L-FABP following fHb infusion indicated hepatocyte damage ($p < 0.01$; **Figure 9.2B**). Loss of intestinal wall integrity after fHb administration was demonstrated by increased ileal leakage of HRP ($p < 0.01$; **Figure 9.2C**) and augmented translocation of luminal bacteria to mesenteric lymph nodes, liver and spleen ($p < 0.001$; **Figure 9.2D**).

Previous studies identified the conversion of hemoglobin and NO to methemoglobin and nitrate and the subsequent decrease of NO bioavailability as a principal cause of hemolysis-related organ damage.^{1,6} Therefore, the effect of pre-exposure of fHb to NO gas on fHb-induced loss of organ integrity was investigated. Spectrophotometrical

analysis demonstrated that freshly generated fHb was oxygenated for 75%. Pre-saturation with NO gas reduced the fHb oxygenation level to 51% and increased methemoglobin levels from 0.3% to 49%. Administration of fHb that was pre-saturated with NO resulted in a significant reduction of renal, hepatic and intestinal compromise compared with administration of fHb that was not saturated with NO (NAG $p < 0.05$; L-FABP $p < 0.05$; HRP $p < 0.01$; bacterial translocation $p < 0.01$; **Figure 9.2A-D**), suggesting a crucial role for NO scavenging in the development of fHb-related organ compromise.

Figure 9.2 Increased levels of circulatory cell-free hemoglobin induce loss of organ integrity via nitric oxide scavenging. fHb infusion resulted in renal (A) and hepatic (B) cell damage and increased translocation of HRP (C) and bacteria (D) through the intestinal wall. Administration of fHb that was pre-saturated with NO prevented renal (A) and hepatic (B) cell damage and improved gut wall barrier (C-D) compared with animals that were infused with unsaturated fHb. * $p < 0.05$; # $p < 0.01$; † $p < 0.001$.

Abbreviations: NAG, N-acetyl- β -D-glucosaminidase; fHb, free haemoglobin; L-FABP, liver fatty acid binding protein; HRP, horseradish peroxidase; NO, nitric oxide.

Protective nutritional effects on organ integrity following acute hemolysis are mediated by peripheral cholecystokinin-1 receptors and nicotinic acetylcholine receptors. Enteral lipid-rich nutrition strongly decreased urinary NAG levels following fHb infusion compared with fasted controls, indicating a reduction of renal cell damage ($p < 0.05$; **Figure 9.3A**). Next, hepatocyte damage as assessed by circulating LFABP levels was significantly reduced by nutritional intervention ($p < 0.05$; **Figure 9.3B**). In previous studies, the intestine was identified as an early target organ of nutritional vagus activation.³¹ Here, pre-treatment with lipid-rich nutrition significantly reduced gut leakiness to HRP ($p < 0.01$; **Figure 9.3C**) and bacterial translocation ($p < 0.05$; **Figure 9.3D**) following fHb infusion, indicating preservation of intestinal integrity by lipid-rich nutrition.

To investigate the involvement of the CCK-1R-dependent vagal pathway in the nutritional impact on hemolysis-induced loss of organ integrity, an antagonist (A70104) and an agonist (PEG-CCK9) to peripheral CCK-1R were applied. Administration of

A70104 abrogated the protective effects of lipid-rich nutrition on hepatic and intestinal integrity (all $p < 0.05$; **Figure 9.3B-D**), but did not significantly affect the nutritional inhibition of renal damage. Next, PEG-CCK9 decreased urinary NAG levels, ameliorated leakage of HRP through the gut wall and reduced bacterial translocation in fasted animals compared with vehicle (all $p < 0.05$; **Figure 9.3A,C,D**). Furthermore, a trend towards reduced hepatocyte damage was observed in fasted animals receiving PEG-CCK9 ($p = 0.08$; **Figure 9.3B**).

The role of the vagal reflex was further assessed by administration of chlorisondamine, an antagonist of peripheral nAChR. Chlorisondamine administered prior to fHb abrogated the protective effects of enteral nutrition on renal, hepatic and intestinal organ integrity (all $p < 0.05$; **Figure 9.3A-D**), pointing at a crucial role for nAChR in the protective effects of nutritional intervention.

Figure 9.3 Enteral lipid-rich nutrition (LRN) improves organ integrity after fHb infusion via activation of peripheral cholecystikinin-1 and nicotinic acetylcholine receptors. Renal (A) and hepatic (B) cell damage were reduced by lipid-rich nutrition compared with fasted controls. Also gut wall permeability to HRP (C) and bacterial translocation (D) was significantly reduced in lipid-rich fed animals. The organ-preserving effects of lipid-rich nutrition were abrogated by A70104, an antagonist of peripheral CCK-1R, and mimicked by CCK-1R agonist PEG-CCK9. The nAChR antagonist chlorisondamine abrogated the nutritional impact on hemolysis-induced organ damage. * $p < 0.05$; # $p < 0.01$.

Lipid-rich nutrition reduces systemic inflammation induced by cell-free haemoglobin

Infusion of fHb resulted in a marked increase of IL-6 plasma levels at 2 hours. Enteral nutrition significantly reduced IL-6 levels (**Figure 9.4**, $p < 0.05$), which is in coherence with previous studies on the anti-inflammatory effects of short-term intervention with enteral nutrition.¹³ Next, the importance of CCK-1R in the nutritional impact on haemolysis-induced inflammation was measured. CCK-1R antagonist A70104 abrogated the nutritional anti-inflammatory effects ($p < 0.05$), whereas CCK-1R agonist PEG-CCK9 reduced IL-6 levels in fasted animals ($p < 0.05$). Next to crucial involvement of the CCK-1R, also the nAChR was shown to be critical, since nAChR antagonist chlorisondamine abrogated the anti-inflammatory effects of lipid-rich nutrition ($p < 0.01$).

Figure 9.4 Systemic inflammation following cell-free hemoglobin infusion is reduced by nutritional intervention. Circulatory IL-6 concentrations are reduced by enteral lipid-rich nutrition (LRN). CCK-1R antagonist A70104 and nAChR antagonist abrogated the nutritional effects, whereas CCK-1R agonist PEG-CCK9 reduced inflammation in fasted animals. * $p < 0.05$; # $p < 0.01$. Abbreviations: IL, interleukin; veh, vehicle; chlor, chlorisondamine.

Nutritional intervention improves renal, hepatic and intestinal perfusion during acute hemolysis

Since impaired perfusion as a consequence of NO scavenging is considered critical in the development of organ injury during hemolysis, next organ perfusion was assessed following nutritional intervention. In fasted animals, organ perfusion tended to be decreased in the liver and ileum compared with pre-fHb values, whereas renal perfusion was unaffected. Enteral nutrition significantly improved renal and intestinal perfusion at 30 and 60 minutes (all $p < 0.05$; **Figure 9.5AC**) and hepatic perfusion at 60 minutes ($p < 0.05$; **Figure 9.5B**). A trend towards improved hepatic perfusion was observed at 30 minutes ($p = 0.06$). Administration of CCK-1R antagonist A70104 abrogated the nutritional impact on hepatic perfusion at 30 and 60 minutes (both

$p < 0.01$) and ileal perfusion at 30 minutes ($p < 0.05$). Next, a trend towards a significant perfusion reduction by A70104 was seen in the kidneys (30 minutes: $p = 0.09$ and 60 minutes: $p = 0.06$) and at 60 minutes in ileum ($p = 0.06$). Chlorisondamine prevented the effects of lipid-rich nutrition on renal perfusion ($p < 0.05$ at 30 and 60 minutes; **Figure 9.5A**), but did not affect the improvement of hepatic and intestinal perfusion by lipid-rich nutrition (**Figure 9.5B-C**). In fed animals that received chlorisondamine, hepatic and intestinal perfusion was significantly increased compared with fasted animals and lipid-rich treated animals that received A70104 ($p < 0.05$ at 30 and 60 minutes; **Figure 9.5BC**). At 120 minutes, no differences were observed between groups.

Figure 9.5 Enteral nutrition improves splanchnic perfusion. **A.** Lipid-rich nutrition improved renal perfusion at 30 and 60 minutes. Nicotinic acetylcholine receptor antagonist chlorisondamine (chl) abrogated the nutritional effects. **B.** Nutritional intervention improved hepatic perfusion at 60 minutes. A70104 (A7) but not chlorisondamine abrogated the nutritional impact. **C.** Ileal perfusion was significantly increased in lipid-rich treated animals at 30 and 60 minutes. A70104 inhibited the nutritional impact at 30 minutes. Chlorisondamine did not affect hepatic perfusion in fed animals. **D.** The mean arterial pressure decreased moderately during and following HFb infusion. Blood pressure in animals receiving chlorisondamine was reduced compared with other groups at all time points. * $p < 0.05$ LRN vs. fasted; # $p < 0.05$ LRN vs. LRN + A70104; † $p < 0.05$ LRN + chlorisondamine vs. fasted; ‡ $p < 0.05$ LRN + chlorisondamine vs. LRN + A70104.

Next, we investigated whether the observed alterations of organ perfusion were reflected in changes in systemic blood pressure. Continuous fHb infusion resulted in a moderate decrease of the mean arterial pressure after 2 hours (fasted: $p < 0.05$, lipid-rich: $p = 0.07$, lipid-rich + A70104: $p = 0.09$ and lipid-rich + chlorisondamine: $p < 0.01$; **Figure 9.5D**). No differences in mean arterial pressure were observed between fasted and lipid-rich fed animals. Chlorisondamine significantly depressed blood pressure at all time points in animals that received lipid-rich nutrition (**Figure 9.5D**) or were fasted (data not shown), which is in line with previous reports.³⁵

DISCUSSION

Intravascular hemolysis, a common phenomenon at intensive care units, is growingly recognized as an independent source of tissue damage and poor clinical outcome.^{6,36} The current manuscript is first to demonstrate protective effects on organ integrity by stimulation of the vagal anti-inflammatory reflex with lipid-rich nutrition in a setting of acute hemolysis.

In the current study, hemolysis was simulated in rodents by infusion of cell-free hemoglobin derived from lysed erythrocytes.¹ The obtained median plasma level of 43 μM is comparable to the fHb concentrations following cardiovascular surgery in which a cardiopulmonary bypass is used or in patients suffering from a sickle cell crisis or paroxysmal nocturnal hemoglobinuria.^{2,37-39} Furthermore, these levels surpassed the critical thresholds of 6 μM fHb, reported to produce vasoconstriction in sickle cell patients⁵, and 10 μM fHb, associated with renal damage in patients undergoing aorta repair surgery.² The current model mimics the clinical setting of hemolysis since not only fHb but also other constituents from lysed erythrocytes were infused. Within 2 hours, this procedure led to rapid compromise of renal, hepatic and intestinal integrity.

Here, short-term intervention with enriched enteral nutrition was demonstrated to reduce renal and hepatic cell damage and preserve gut barrier function shortly following acute hemolysis. The applied feeding regimen and nutritional composition were previously shown to be protective in several experimental models of acute inflammation, including hemorrhagic shock, bacterial DNA exposure and postoperative ileus.^{13,17,40} In addition, the present study demonstrates that lipid-rich nutrition significantly reduces hemolysis-induced systemic inflammation, assessed as circulatory IL-6 concentrations. Critical involvement of the vagal anti-inflammatory reflex was demonstrated by agonists and antagonists of peripheral CCK-1R (A70104 and PEG-CCK9 respectively). The finding that nAChR antagonist chlorisondamine abrogated the nutritional effects on hemolytic damage provides further evidence for the vagal anti-inflammatory reflex as the underlying mechanism. In addition to nutritional

intervention, the vagal pathway has also been stimulated by pharmacological and electrical means in various experimental settings¹⁹, however to our knowledge not in the context of acute hemolysis.

During massive hemolysis, the pool of endogenous scavengers of cell-free hemoglobin such as haptoglobin rapidly becomes depleted.⁴¹ Subsequent NO scavenging by oxygenated hemoglobin is considered pivotal in the development of organ damage in hemolytic disease.^{1,6} The importance of NO scavenging is confirmed in the current rodent hemolysis study, as pre-saturation of fHb with NO gas, resulting in decreased oxygenated hemoglobin levels and enhanced methemoglobin formation, considerably reduced organ damage compared with animals that received non-saturated fHb. In line with our findings, saturation of circulatory fHb with inhaled NO was shown to improve tissue perfusion in a canine hemolysis model.¹

Using fluorescent microspheres, lipid-rich nutrition was demonstrated to improve renal, hepatic and intestinal perfusion after hemolysis compared with fasted controls. The alterations of organ perfusion were not reflected by changes in mean arterial pressure. Over two hours, a gradual and moderate decrease of mean arterial pressure was observed in both fasted and fed animals, which is conform observations in a model of sickle cell disease.⁸ CCK-1 receptor antagonist A70104 abrogated the nutritional effects on renal, hepatic and intestinal perfusion, indicating that the nutritional improvement of organ perfusion was transferred by the vagal anti-inflammatory reflex. Since CCK has also been reported to improve mesenteric blood flow via non-neural pathways^{42,43}, we investigated whether nicotinic receptor antagonist chlorisondamine was able to blunt the nutritional impact on organ perfusion. The finding that chlorisondamine abrogated the nutritional effect on renal perfusion is consistent with a previous report on the markedly increased nAChR expression on the endothelium of peritubular capillaries and the decrease of tubular damage following administration of cholinergic agonists in a setting of renal ischemia and reperfusion⁴⁴. Interestingly, although chlorisondamine effectively abrogated the effects of lipid-rich nutrition on hepatic and intestinal integrity, it did not affect the nutritional effects on intestinal and hepatic perfusion. Therefore, based on the data of the present study, the protective potential of lipid-rich nutrition cannot be attributed to the improvement of organ perfusion. Arguably, the protective effects on organ integrity in the present hemolysis setting may be caused by direct vagal inhibition of the local inflammatory response.^{45,46}

A range of interventions aimed at reducing hemolysis-related organ damage is currently under investigation.³⁶ The present study indicates that enteral administration of limited quantities of a well-designed nutritional composition, being a physiological approach, may be of additional value to limit the detrimental sequelae of hemolysis. Such nutritional intervention fits in the current guidelines and recommendations that

favor early start of enteral feeding in critical care and surgical patients.^{47,48} Since also post-treatment with lipid-rich nutrition was previously shown to attenuate inflammation and preserve organ integrity, further studies should clarify whether nutritional intervention is also applicable when hemolysis is already manifested.³² In addition, the findings of this study may be relevant for polytrauma settings considered the shared NO-scavenging properties between hemoglobin and myoglobin, the later being massively released following myocyte damage.⁴⁹

In summary, lipid-rich nutrition preserves renal, hepatic and intestinal integrity and reduces systemic inflammation in a CCK-1R and nAChR-dependent manner in a rodent model of acute hemolysis. In addition, nutritional intervention significantly improves perfusion of these organs during hemolysis. These data expand the potential applicability of nutritional activation of the vagal anti-inflammatory reflex to patients prone to develop hemolysis.

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Summary and discussion

10

A grayscale microscopic image of several red blood cells, showing their characteristic biconcave disc shape. The cells are in focus, with some appearing more prominent than others. The background is dark and slightly blurred, emphasizing the cells.

Prevention and limitation of tissue damage is desirable during every surgical procedure as organ injury is independently associated with adverse patient outcome. In the setting of (complex) cardiovascular surgery, the development of intestinal mucosal injury and renal tubular damage is frequently reported and associated with increased morbidity and mortality.¹⁻¹⁶ Insight into the pathophysiological mechanisms underlying the development of tissue damage is essential for the development of specific treatment and prevention strategies in this setting.

The cardiopulmonary bypass (CPB) is considered to play an important role in the development of tissue injury during cardiovascular surgery. CPB-induced ischemia-reperfusion injury, hypoperfusion injury, enhanced coagulation, inflammation, and hemodilution are all considered to play a role in impaired organ perfusion and tissue injury development during surgery.¹⁷⁻²³ CPB also leads to red blood cell injury and hemolysis, causing the release of hemoglobin into the circulation.²⁴ The adverse effects of hemolysis on microcirculatory blood flow and organ compromise has gained increasing interest in patients with chronic hemolytic disease as it was found that cell-free hemoglobin (fHb) is a potent scavenger of intravascular nitric oxide (NO).²⁵⁻²⁷ The fHb-induced decrease in NO-bioavailability has been related to increased endothelial dysfunction, decreased (microcirculatory) blood flow, and tissue injury in both animal models and human studies.^{25,26,28} Hemolysis is a known consequence of cardiopulmonary bypass, however the impact of increased fHb concentrations on tissue injury development in the setting of on-pump cardiovascular surgery is not well studied. This thesis aimed to investigate whether hemolysis contributes to the development of intestinal and renal injury during and after cardiovascular surgery, and, in addition, if scavenging of intravascular NO played a pathophysiological role in this association.

The **first aim** of this thesis was to investigate the extent of intestinal and renal injury development during (on-pump) aortic surgery and cardiac surgery. Damage to the intestinal epithelium and renal tubular cells is of clinical importance. Intestinal tissue damage has been frequently associated with the development of the systemic inflammatory response syndrome (SIRS), sepsis, and multiple organ failure.^{29,30} Loss of intestinal wall integrity facilitates translocation of microbiota and microbial-derived toxins from the gut lumen to the systemic circulation where several potent signaling cascades are triggered (NF- κ B, AKT-phosphatidylinositol-3'-kinase, and mitogen activated protein kinase pathways). This subsequently results in the release of pro-inflammatory substances like interleukin 6 (IL-6), IL-8, and TNF- α , cytokines which are considered to be key players in the development of the systemic inflammatory response.³¹⁻³⁶ Also, damage to the (proximal) renal tubular epithelium has been independently correlated with increased morbidity. Postoperative acute kidney injury

(AKI) was reported to be the strongest postoperative risk factor for death (odds ratio of 7.9) in patients undergoing cardiac surgery.^{37,38} However, not only gross changes in postoperative renal function but even small alterations of serum creatinine have been independently linked to adverse outcome in this setting, indicating the importance of preventing postoperative renal dysfunction after cardiovascular surgery.³⁹

Using plasma intestinal fatty acid binding protein (IFABP), a sensitive and specific marker of intestinal mucosal injury⁴⁰⁻⁵¹, and urinary N-acetyl- β -D-glucosaminidase, a marker of proximal tubular epithelial damage⁵², we were able to demonstrate the development of intestinal mucosal injury and renal tubular damage in patients undergoing open surgical repair of an abdominal aortic aneurysm (OR-AAA) or thoraco(abdominal)aortic aneurysm (OR-TAA(A)) in *Chapter 2*, *Chapter 3*, and *Chapter 6*, and in patients subjected to on-pump cardiac surgery (coronary artery bypass grafting (CABG), and combined CABG and valve surgery (CABG+Valve)) in *Chapter 7*. In addition, the extent of mucosal injury appeared to be significantly correlated to the increase of plasma IL-6 and IL-8 concentrations in patients undergoing OR-TAA(A) surgery (*Chapter 3*), supporting the previously stated association between intestinal damage and the systemic inflammatory response.

Next to the fact that plasma IFABP was able to indicate intestinal mucosal injury (present vs. not-present) during cardiovascular surgery, the total increase in IFABP levels also provided valuable information on the extent and severity of intestinal mucosal injury (*Chapter 2*). Patients developing lethal postoperative intestinal necrosis displayed exceptionally high levels of plasma IFABP already at the end of surgery. In fact, determination of plasma IFABP enabled identification of patients with postoperative intestinal necrosis one to two days prior to the clinical diagnosis with high sensitivity and specificity. Tissue hypoperfusion and ischemia, with subsequent inadequate tissue oxygenation, is considered to play a crucial role in the development of (intraoperative) intestinal tissue damage and intestinal barrier loss.^{53,54} Our observation that low arterial pH and increased plasma lactate concentrations, suggestive of tissue hypoxia⁵⁵, had additional value in the identification of patients with transmural intestinal necrosis after OR-AAA surgery, supports this theory. Furthermore, the fact that patients subjected to on-pump cardiovascular surgery (OR-TAA(A), CABG, and CABG+Valve surgery) displayed highest IFABP levels during surgery suggests more profound visceral perfusion derangements in these patients, consistent with previous reports on the adverse effect of CPB on splanchnic perfusion.^{14,17,18} Regarding renal outcome, the perioperative course of urinary NAG displayed similar kinetics to plasma IFABP; with highest levels measured in patients subjected to on-pump cardiovascular surgery. Furthermore, patients with postoperative AKI displayed

(a trend towards) increased in-hospital mortality and hospitalization time (*Chapter 6 and 7*) compared to non-AKI patients.

Summarizing, these results indicate the clinical importance of prevention of intestinal mucosal injury, intestinal necrosis, renal tubular damage and AKI-development during and after cardiovascular surgery. Patients undergoing on-pump surgery appear to be at highest risk for these complications. In contrast, patients undergoing endovascular exclusion of an abdominal aortic aneurysm (EVAR) or off-pump CABG (OPCAB) did neither display significant increases in plasma IFABP nor urinary NAG during the perioperative period. Although this is in line with many clinical observations⁵⁶⁻⁵⁸, also contrary results have been published reporting on a similar or even increased risk of postoperative intestinal or renal complications in EVAR or OPCAB patients compared to patients requiring on-pump surgery.^{10,59-61}

In 2002, Reiter and co-workers published a landmark paper on the adverse effect of hemolysis on microcirculatory perfusion and fore-arm blood flow in patients with sickle cell disease, a chronic hemolytic disorder.²⁵ Release of the hemolytic products fHb and arginase-1 from the erythrocyte was suggested to impair NO-bioavailability (the most important regulator of vascular tone) through intravascular NO-scavenging and degradation of the NO-substrate arginine, respectively. Since then, a series of papers substantiated this observation and provided additional evidence for the potent NO-scavenging property of fHb. Release of fHb during intravascular hemolysis is now believed to underlie the adverse effect of hemolysis on tissue perfusion and patient outcome in several acquired and hereditary (chronic) hemolytic diseases.^{26-28,62-70}

Hemolysis is a relatively common phenomenon during cardiovascular surgery and generally attributed to the CPB^{24,71}, cell-saver⁷², and (massive) red blood cell transfusions^{73,74}. Increased fHb levels have been postulated to contribute to renal injury through intratubular release of free heme and free iron after glomerular filtration of fHb.⁷⁵ In contrast, the effects of hemolysis on the intravascular NO-metabolism in this setting has, to our knowledge, not been studied before. The **second aim** of this thesis was therefore to investigate the extent of acute hemolysis during cardiovascular surgery, in addition to the effects of increased plasma fHb levels on the intravascular NO-metabolism. *Chapter 4* elaborately discusses the potential sources and effects of hemolysis during cardiovascular surgery. Also, several therapeutic options to attenuate the adverse effects of hemolysis are highlighted. *Chapter 5*, *Chapter 6*, and *Chapter 7* demonstrate the development of hemolysis, indicated by increased plasma fHb, in patients undergoing on-pump OR-TAA(A), and cardiac surgery. In addition, arginase-1 levels were analyzed in OR-TAA(A) patients in *Chapter 5*. Fhb and arginase-1 levels increased significantly after initiation of CPB, peaking at

the end of CPB to two hours after cessation of CPB. The impact of the CPB on the induction of hemolysis was demonstrated by inclusion of a group of patients undergoing off-pump coronary surgery (OPCAB) who did not show significant changes in fHb. Furthermore, perfusion time was significantly correlated to both peak fHb levels and total release of fHb during the study period (estimated using the area under the curve), as was consistent with previous reports on CPB-induced hemolysis.^{24,76}

The total release of fHb and the arginase-1 levels correlated significantly. However, the increase in arginase-1 levels was not sufficient to significantly alter the ratio between arginine and ornithine plasma levels. This observation suggests that arginase-1 release does not reduce arginine availability, and therefore does not (significantly) contribute to a reduction of NO-bioavailability during cardiovascular surgery. These results were in line with the results of van de Poll et al, who did not find a significant effect of increased arginase-1 levels (mean levels 185 ng/ml which was comparable to concentrations found in this study) on circulating arginine levels during liver resection.⁷⁷ Using in vitro experiments, they concluded that arginase-1 levels had to exceed 2000 ng/ml to significantly reduce plasma arginine concentrations.⁷⁷ In contrast, Morris et al reported on a significant reduction of the arginine/ornithine ratio in 209 patients with sickle cell disease (compared to 36 controls) which was significantly (inversely) correlated with increased plasma arginase-1 activity.⁶⁹ The fact that actual fHb and arginase-1 levels were not reported prevented us however from comparing these findings with the presented results in *Chapter 5*. The contribution of arginase-1 release to decreased NO-bioavailability during cardiovascular surgery remains therefore to be further clarified.

In our patients, we observed mean (peak) fHb levels varying between 10 $\mu\text{mol/l}$ and 25 $\mu\text{mol/l}$. In the literature, fHb levels over 6 $\mu\text{mol/l}$ are considered to be (clinically) relevant as these levels have been associated with significant derangements in the NO-metabolism in animal studies and in patients.^{25,78} Considering this “threshold” level, we hypothesized that the increased fHb levels as observed in our patients would (significantly) impair NO-bioavailability as well. To that end, plasma NO-consumption was studied in patients undergoing cardiac surgery. *Chapter 7* describes the significant increase in plasma NO-consumption during surgery in patients subjected to CABG or CABG+Valve surgery. Consistent with the NO-scavenging property of fHb, the total release of fHb was significantly correlated to total plasma NO-consumption. NO-consumption increased 2.4-fold in CABG-patients, and almost 8-fold in CABG+Valve patients, in whom NO-consumption levels of 266 $\mu\text{mol/l}$ per liter of plasma were observed. As levels of plasma NO consumption of 10-15 $\mu\text{mol/l}$ have been related to a significant impairment of NO-dependent fore-arm blood flow, we consider the extent of NO-consumption in these patients to be clinically relevant.^{25, 79} Patients undergoing

OPCAB surgery did not display significant changes in plasma NO-consuming capacity, which was in line with the absence of hemolysis in this patient group. Plasma NO-consumption was not analyzed in patients subject to OR-TAA(A) surgery. Nevertheless, as plasma fHb concentrations in these patients corresponded to fHb levels measured in CABG+Valve surgery patients, we expect the level of NO-consumption to be similar.

The impact of increased plasma fHb levels, and the subsequent increase in plasma NO-consuming capacity, on NO-mediated vasodilation was studied in *Chapter 5*. Using fore-arm venous occlusion plethysmography (a technique widely used to non-invasively study (arterial) vascular function in vivo⁸⁰) blood flow responses to intra-arterial infusion of the NO-donor sodium nitroprusside (SNP) were studied. Infusion of acetylcholine (Ach, a partly NO-independent but endothelium-dependent vasodilator) was performed to control for impaired endothelial vasodilatory capacity. We hypothesized that presence of fHb in the blood compartment would compete for the NO moiety released from SNP, preventing its vasodilatory effect. Interestingly, blood flow responses to SNP during peak fHb levels were significantly reduced compared to SNP-induced vasodilation on the first postoperative day when plasma fHb concentrations had normalized, indeed suggesting (although indirectly) scavenging of SNP-derived NO by fHb. Acetylcholine-induced vasodilation did not differ between the studied time points, indicating unchanged vasodilatory capacity of the brachial artery. In conclusion, acute hemolysis during cardiovascular surgery correlates to enhanced plasma NO-consumption capacity and reduces NO-dependent vasodilation of the (forearm) vasculature. These results support, but do not prove, a causal relation between increased plasma fHb levels, enhanced NO-scavenging and impaired blood flow in patients subjected to on-pump cardiovascular surgery.

Derangements in (microcirculatory) tissue perfusion play a central role in tissue injury development, especially when coinciding with a pro-inflammatory state as is often the case during (on-pump) cardiovascular surgery.^{41,81-83} Considering the data discussed above, the **third aim** of this thesis was to investigate whether acute hemolysis is associated with the development of visceral injury during cardiovascular surgery. As presented in *Chapter 7*, total release of fHb was significantly correlated with intestinal mucosal injury and renal tubular injury during on-pump cardiac surgery (CABG and CABG+Valve surgery). Similarly, a significant correlation between hemolysis and tubular injury was found in patients undergoing OR-TAA(A) surgery (*Chapter 6*). The correlation between fHb and intestinal damage was not assessed in these patients. The correlation between plasma NO-consumption, plasma IFABP concentrations, and urinary NAG values, was comparable to the correlation between fHb, IFABP, and NAG substantiating a potential mechanistic role of fHb-induced NO-scavenging in the

development of visceral tissue injury in this setting. In addition to a positive correlation between hemolysis and subclinical renal tubular damage (indicated by increased urinary NAG values), it was found that patients with postoperative AKI (after OR-TAA(A) or cardiac surgery) displayed significantly higher plasma fHb levels during surgery compared to non-AKI patients, indicating that substantial hemolysis may be a direct risk factor for postoperative AKI as well. Considering the fact that hemolysis correlated with tissue damage of two independent organ systems, it is tempting to speculate that fHb can also induce tissue injury in other organs, for instance in the heart, liver, lungs.^{68,84}

Historically, hemolysis has been principally contributed to the CPB.^{24, 76, 85-87} In line, we were able to indicate the CPB as the principal source of fHb during cardiovascular surgery (*Chapter 5*), with CPB-duration being significantly correlated to both peak fHb levels and total release of fHb during the study period (*Chapter 7*). Nevertheless, the results presented in Chapter 5 also suggest that cell-saver and transfusion of stored red blood cells (RBC) contribute to increased plasma fHb and arginase-1 levels, albeit to a lesser extent. The observation that transfusion of stored RBC may significantly contribute to increased plasma fHb concentrations and arginase-1 levels was interesting in light of the increasing number of papers reporting on the adverse relation between blood transfusion and adverse patient outcome.^{74, 88-93} The **fourth aim** of this thesis was therefore to study the contribution of stored RBC transfusion on circulating fHb levels and plasma NO-consumption in patients (*Chapter 8*). To that end, we studied a group of patients undergoing isolated transfusion of stored RBC (1 or 2 units), that way preventing the confounding influence of the CPB and cell-saver. Indeed, transfusion of 2 stored RBC units significantly increased circulating fHb levels and plasma NO-consumption as soon as 15 minutes after transfusion. Transfusion of 1 stored RBC unit did not evoke a comparable increase of fHb or reduction in NO-bioavailability. The current debate concerning the relation between blood transfusion and adverse outcome mainly focuses on the effects of RBC storage duration. As RBC damage increases with prolonged storage, it has been suggested that transfusion of “old blood” is more harmful compared to “young blood”.⁷⁴ Interestingly, we found no correlation between storage duration of the transfused units, the increase in plasma fHb, and NO-consumption. However, an inverse association between pre-transfusion plasma Hp levels and post-transfusion plasma fHb concentrations was found. This may suggest that patients with high Hp levels may be better protected against the adverse effects of increased fHb levels, potentially through enhanced fHb clearance by Hp, compared to individuals with low baseline Hp concentrations. These results may be of particular importance for patients that receive multiple RBC transfusion, as is particularly seen during OR-TAA(A) and to a lesser extent during CABG+Valve or CABG

surgery. The contributory effect of packed RBC transfusion on circulating fHb levels as demonstrated in *Chapter 8* may even be an underestimation. It has been recently shown that a reduction of the membrane of stored red blood cells results in release of fHb-containing microvesicles which possess an equally potent NO-scavenging property compared to fHb molecules.⁹⁴ Formation and transfusion of these microvesicles were not evaluated in our study.

These results indicate that transfusion of stored RBC units may increase circulating plasma fHb levels and plasma NO-consumption during cardiovascular surgery, thereby contributing to the development of visceral injury. Furthermore, patients with low (pre-operative) plasma Hp concentrations and/or requiring massive blood transfusion, may be at particularly high risk.

Cardiovascular surgery is associated with a significant reduction of circulating Hp levels (*Chapter 7*) during surgery, potentially through increased clearance of Hp-fHb complexes due to intravascular hemolysis. Extrapolating our previous discussed results, we hypothesize that patients with low preoperative Hp levels, preoperative impaired kidney function, undergoing complex cardiovascular surgery associated with long perfusion times and frequent RBC transfusion, are at higher risk for the adverse effects of circulating fHb compared with patients with normal to high preoperative Hp, normal renal function, that undergo CABG or OPCAB surgery, for instance. (Routine) Evaluation of preoperative Hp status may therefore be a worthy addition to the current preoperative patient-risk assessment prior to (complex) cardiovascular surgery. Furthermore, as RBC units with a longer storage time generally contain higher fHb concentrations^{95,96}, it could be speculated that “younger” RBC units should be reserved for patients with lowest preoperative plasma Hp levels, that way limiting the fHb load administered to these patients. Lastly, the administration of fresh frozen plasma during surgery, which contains haptoglobin, might also be worth considering.

The final and **fifth aim** of this thesis was to study the effect of enteral high fat feeding in a rodent model of acute hemolysis with the following rationale: 1) it has been suggested that the vascular reactivity to (partly) NO-independent vasodilatory compounds is (compensatory) increased during hemolysis, 2) acetylcholine (ACh), the principal neurotransmitter of the autonomic vagal nerve, is a much studied (largely) NO-independent vasodilator, 3) enteral lipid-rich nutrition has been shown to potently stimulate vagal activity and release of ACh, and 4) administration of a high fat diet reduced visceral organ injury and (systemic) inflammation in animal models⁹⁷. In an established rodent model of acute hemolysis⁹⁸ (in which similar fHb levels are achieved as measured during on-pump cardiovascular surgery) we found that high fat feeding indeed significantly reduced hemolysis-induced loss of intestinal integrity, renal tubular damage, hepatic cell damage, and systemic inflammation (*Chapter 9*). As lipid-

rich nutrition promoted intestinal, renal and hepatic perfusion during hemolysis, the protective effect of high-fat feeding may indeed be a consequence of Ach-induced vasodilation, though we did not show this in the current study. Alternatively, enteral feeding has been shown to directly increase splanchnic perfusion (reactive postprandial hyperemia) to facilitate the increased oxygen demand.⁹⁹ Either way, early enteral nutrition has been shown to be generally well-tolerated, beneficial for the preservation of intestinal integrity and stimulation of gastro-intestinal blood flow¹⁰⁰, allowing a more rapid return of gastro-intestinal function and reduced morbidity and hospital stay following major gynecologic surgery¹⁰¹, a caesarian section¹⁰², or gastrointestinal resection¹⁰³.

Inferring from these results, it may be hypothesized that administration of high fat enteral nutrition could reduce the adverse effects of hemolysis during CPB-assisted cardiovascular surgery.

CONCLUSION

In the current thesis, we showed that hemolysis significantly decreased intravascular NO-bioavailability and impaired NO-mediated vasodilation during and after CPB-assisted cardiovascular surgery. Furthermore, enhanced plasma fHb concentrations correlated with the development of intestinal mucosal injury, renal proximal tubular damage and clinical acute kidney injury in this setting. Low pre-operative haptoglobin levels, in addition to (massive) transfusion of stored RBC units during surgery, may further enhance the patients' susceptibility to the adverse effects of hemolysis. Considering these data, we consider that hemolysis significantly contributes to increased morbidity and mortality after cardiovascular surgery. As the adverse effects of fHb can be relatively easily counteracted *in vivo*, we consider that hemolysis is an attractive and promising (new) therapeutic target to improve patient outcome after cardiovascular surgery.

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Nederlandse samenvatting

Hart- en vaatziekten (cardiovasculaire ziekten) vormen nog steeds de primaire doodsoorzaak in Nederland. Voor een aantal hart- en vaataandoeningen is operatief ingrijpen de enige behandeling. Voorbeelden van dergelijke ziekten zijn het aorta aneurysma (een abnormale verwijding van de lichaamsslagader), coronair vaatlijden (meestal ten gevolge van vernauwingen van de kransslagaders van het hart) en hartklep afwijkingen. Tijdens dergelijke operaties moet vaak de functie van het hart en longen tijdelijk (buiten het lichaam) worden overgenomen. De ontwikkeling van de hart-longmachine (cardiopulmonale bypass of CPB) in het begin van de jaren vijftig van de vorige eeuw heeft dan ook een grote vooruitgang betekend in de behandelingsmogelijkheden van deze patiënten.

Tot op de dag van vandaag wordt de CPB nog veelvuldig gebruikt bij hart operaties en enkele grote aorta operaties. De enorme technische ontwikkeling binnen de hart- en vaatchirurgie, met name op het gebied van de CPB, heeft gezorgd voor een flinke vermindering van het aantal postoperatieve complicaties en de sterftetekans (mortaliteit). Desalniettemin komen, vooral na meer complexe aorta- en hartoperaties, nog relatief veel postoperatieve complicaties voor. Voorbeelden van dergelijk complexe operaties zijn het herstel van een thoracoabdominal aorta aneurysma (een uitgebreid aorta aneurysma waarbij zowel de aorta in de borstholte (de thorax) als in de buikholte (het abdomen) verwijdt is), en hartoperaties waarbij zowel omleidingen (bypasses) rond zieke coronair arteriën worden aangelegd (een coronary artery bypass grafting of CABG) en bovendien aan één of meerdere hartkleppen geopereerd moet worden. Vaak gerapporteerde postoperatieve complicaties na deze operaties zijn: stoornissen van de functie van de nier (tot dialyse aan toe), darmproblemen, longcomplicaties en hartproblemen. Ook wordt frequent een versterkte respons van het immuunsysteem waargenomen, wat zich klinisch uit in een verhoogde concentratie van ontstekingsmarkers in het bloed (een systemische inflammatoire respons). Deze ontstekingsrespons kan zich dusdanig uitbreiden dat dit leidt tot sepsis en het falen van meerdere organen. Eerder onderzoek in proefdieren en patiënten heeft aangetoond dat de mate van deze ontstekingsrespons gerelateerd is aan de mate van darmschade. Schade aan de darm barrière faciliteert namelijk (o.a.) de verplaatsing van bacteriën vanuit de darm naar de bloedbaan. De daaropvolgende reactie van het immuunsysteem is er op gericht deze ongewenste indringers zo snel mogelijk op te ruimen. Deze reactie van het immuunsysteem is om deze reden goed voor de patiënt. Een te sterke lokale ontstekingsreactie kan echter soms moeilijk onder controle gehouden worden door het immuunsysteem en uitbreiden tot een systemische inflammatoire respons en schade aan (andere) organen.

Om de kans op het ontwikkelen van postoperatieve complicaties zo laag mogelijk te houden en, indien zich toch een complicatie ontwikkelt, de patiënt zo optimaal mogelijk te kunnen behandelen is het identificeren van *beïnvloedbare* risicofactoren (dus bijvoorbeeld de operatieduur) van belang. Immers, interventies gericht tegen dergelijke risicofactoren kunnen de uitkomst van de patiënt verbeteren. Risicofactoren zoals leeftijd en geslacht van de patiënt zijn niet beïnvloedbaar en kunnen om deze reden dan ook niet gebruikt worden voor therapeutische doeleinden.

De CPB is onvervangbaar en essentieel om sommige operaties uit te kunnen voeren. Desalniettemin heeft gebruik van een CPB ook ongewenste effecten. Eén van deze effecten is hemolyse dat, letterlijke vertaald, het 'uit elkaar vallen (*lysis*) van het bloed (*hemo*)' betekend. In de geneeskundige praktijk wordt deze term echter exclusief gebruikt voor het vroegtijdig kapot gaan van rode bloedcellen in de bloedbaan. Hemolyse ontstaat tijdens CPB vooral door mechanische stress in de slangen en pompen waaruit de CPB is opgebouwd. Tijdens hemolyse komen verschillende stoffen in het bloed terecht die normaal gesproken alleen in de rode bloedcel aanwezig zijn. Voorbeelden hiervan zijn het eiwit hemoglobine (dan vrij hemoglobine of vHb genoemd) en het enzym arginase-1. In 2002 is ontdekt dat zowel vHb als arginase-1 de beschikbaarheid van het molecuul stikstof monoxide (nitric oxide, NO) in het bloed significant beperkt. Dit gebeurt op twee manieren: vHb reageert en inactiveert NO, en arginase-1 leidt tot afbraak van arginine, het aminozuur waaruit NO geproduceerd wordt. Omdat NO de belangrijkste vaatverwijder in ons lichaam is en essentieel is voor (het behoud van) een adequate weefseldoorbloeding, kan een verminderde beschikbaarheid van NO nadelige gevolgen hebben voor de orgaandoorbloeding en zo leiden tot orgaanschade. Bij patiënten met chronisch hemolytische aandoeningen (zoals malaria of sikkel cel anemie) is aangetoond dat hemolyse inderdaad gecorreleerd is met een slechtere doorbloeding van organen en weefselschade. Deze associatie kon verklaard worden door een verminderde beschikbaarheid van NO.

Hemolyse is ook tijdens chirurgie met CPB een bekend fenomeen, desalniettemin is de relatie tussen hemolyse en orgaanschade in deze setting nog beperkt bestudeerd. Wij hebben ons in dit proefschrift dan ook tot doel gesteld om te onderzoeken of hemolyse tijdens aorta- en hartchirurgie gecorreleerd is met schade aan darm- en nierweefsel (viscerale schade). Viscerale schade heeft een direct negatief effect op de prognose van de patiënt. Darmschade is, zoals boven beschreven, geassocieerd met een te sterke ontstekingsreactie van het lichaam. Nierschade, dat vrij vaak voorkomt, is gerelateerd aan het optreden van andere complicaties en een slechtere postoperatieve overleving. In dit proefschrift hebben wij vervolgens onderzocht of de eventuele relatie tussen hemolyse en viscerale schade verklaard kan worden door een verminderde beschikbaarheid van NO.

Het **eerste doel** van dit proefschrift was het vaststellen van de mate van darm- en nierschade tijdens bepaalde typen aorta- en hartoperaties (**Hoofdstukken 2, 3, 6, en 7**). Dit werd onderzocht door bepaling van het eiwit intestinal fatty acid bindig protein (IFABP), een gevoelige marker voor schade aan darmcellen, in het bloed en door bepaling van het enzym N-acetyl- β -D-glucosaminidase (NAG), een goede indicator voor nierschade, in de urine. Verhoogde waarden van IFABP en NAG suggereren darm- en nierschade. Darm- en nierschade was het meest uitgesproken in patiënten die complexe operaties met een CPB ondergingen zoals open chirurgisch herstel van een thoracaal of thoracoabdominal aorta aneurysma (OR-TAA(A)), of gecombineerde bypass + hartklep operaties. In tegenstelling tot deze patiënten was darm- en nierschade zeer beperkt in patiënten die werden geopereerd aan een geïsoleerd aorta aneurysma in de buik (een abdominaal aorta aneurysma, hierbij wordt geen CPB gebruikt) en zelfs onmeetbaar bij patiënten die een hartoperatie zonder CPB of een aortaoperatie via de lies ondergingen.

Hoewel de patiënt deze mate van orgaanschade niet direct zelf hoeft te merken, heeft eerder onderzoek aangetoond dat deze zogenaamde *subklinische* schade (waarmee bedoeld wordt dat we de schade wel in het bloed of in urine kunnen aantonen) wel degelijk de kans op het ontwikkelen van complicaties vergroot en de overlevingskans van de patiënt verlaagt. In lijn met dit eerdere onderzoek bleek bij patiënten met (sterk) verhoogde NAG en vHb spiegels tijdens de operatie vaker postoperatieve nierfunctiestoornissen op te treden. Sommige patiënten werden zelfs tijdelijk dialyseafhankelijk (**Hoofdstuk 6 en 7**). De mate van darmschade bleek samen te hangen met de hoeveelheid ontstekings-eiwitten (interleukine-6, en interleukine-8) in het bloed van de patiënt (**Hoofdstuk 3**). Bovendien voorspelde zeer hoge bloedspiegels van IFABP al vroegtijdig het optreden van darmischemie (het volledig afsterven van een stukje darm, **Hoofdstuk 2**). Deze laatste observatie was van uitgesproken klinisch belang omdat darmischemie een zeer lastig te diagnosticeren fenomeen is. Gezien de ernst van de aandoening, de meeste patiënten overleven darmischemie niet, is dit een groot probleem. Bepaling van IFABP aan het einde van de operatie maakte het mogelijk patiënten met darmischemie vroegtijdig, 1-2 dagen voordat zij ziek werden, te identificeren. Het zal in de toekomst moeten blijken of het gebruik van IFABP in het opsporen van patiënten met darmischemie zal leiden tot snellere behandeling van deze ziekte, en daarmee leidt tot een verbeterde overleving van deze patiënten.

Het **tweede doel** van dit proefschrift was het onderzoeken van de mate van hemolyse en de eventuele invloed van hemolyse op de NO-beschikbaarheid tijdens en na aorta- en hartchirurgie (**Hoofdstuk 4, 5, 6 en 7**). Overeenkomend met de stijging van IFABP en NAG was de stijging van vHb het sterkst in de OR-TAA(A) patiënten en bij complexe hartoperaties. In deze patiënten bereikte de vHb concentraties in het bloed een niveau

dat in eerder onderzoek geassocieerd was met een vermindering van de NO-beschikbaarheid en een vermindering van weefseldoorbloeding. In tegenstelling tot de vHb waarden waren de arginase-1 spiegels tijdens hemolyse in OR-TAA(A) patiënten niet hoog genoeg om een duidelijke afname van arginine te kunnen veroorzaken (**Hoofdstuk 5**). Om de beschikbaarheid van NO te onderzoeken hebben wij het verbruik van NO in het bloed onderzocht; de zogenaamde 'plasma NO-consumptie'. Hoe hoger de NO-consumptie van het plasma, hoe minder NO beschikbaar blijft voor, bijvoorbeeld, het induceren van vaatverwijding. De mate van hemolyse (gemeten door vHb) bleek significant gecorreleerd te zijn aan de mate van NO-consumptie in het bloed, hetgeen overeen kwam met de theorie dat vHb NO wegvangt (**Hoofdstuk 7**). NO handhaaft de weefseldoorbloeding door vaatverwijding te veroorzaken. Als er door aanwezigheid van vHb veel NO wordt weggevangen is dus mogelijk de reactie van de vaatwand op toegediend NO ook verstoort. Om deze reden hebben wij vervolgens in een kleine groep OR-TAA(A) patiënten onderzocht of de vaatwand minder vaatverwijding vertoonde als reactie op het toedienen van de NO-donor sodiumnitroprusside op het moment dat er hoge concentraties vHb in het plasma meetbaar waren (**Hoofdstuk 5**). Dit bleek inderdaad het geval. Wij veronderstellen dat dit een gevolg is van het wegvangen van het, van sodiumnitroprusside afkomstige, NO door het vHb. Controle experimenten een dag later, toen vHb spiegels genormaliseerd waren, liet een sterkere vaatverwijding zien na sodiumnitroprusside toediening. Deze resultaten suggereren dat er minder bloedvatverwijding optreedt in reactie op toegediend NO bij hoge vHb concentraties in het bloed in vergelijking met momenten waarop weinig vHb in het bloed aanwezig is. Deze data sluiten aan op het reeds bekende feit dat vHb zorgt voor inactivatie van NO in de bloedbaan.

Nadat we hadden aangetoond dat er tijdens (complexe) cardiovasculaire chirurgie met CPB zowel darmschade, nierschade, als hemolyse met een significante beperking van de intravasculaire NO-beschikbaarheid optreedt, stelden wij ons tot doel (**derde doel**) om te onderzoeken of deze fenomenen met elkaar verband houden. Hiervoor hebben wij de totale vrijgave van het IFABP, NAG, vHb en de totale mate van plasma NO-consumptie tijdens de studieperiode met elkaar gecorreleerd. In zowel OR-TAA(A) patiënten als hartchirurgie patiënten was hemolyse significant gecorreleerd aan nierschade (**Hoofdstuk 6 en 7**) en aan darmschade (**Hoofdstuk 7**). Ook was de mate van NO-consumptie significant gecorreleerd aan beide schademarkers (**Hoofdstuk 7**). Zelfs na statistische correctie voor andere beïnvloedende factoren bleef de significante relatie tussen hemolyse en viscerale schade bestaan. Piekwaarden van plasma vHb tijdens de operatie hadden tevens een voorspellende waarde voor postoperatieve nierfunctiestoornissen (**Hoofdstuk 6**).

Deze data maken het aannemelijk dat hemolyse gerelateerd is aan de ernst van viscerale schade tijdens (complexe) cardiovasculaire chirurgie met CPB. Tevens is het waarschijnlijk dat een verminderde beschikbaarheid van NO, als gevolg van het wegvangen van NO door vHb, belangrijk is in deze relatie.

Hemolyse tijdens cardiovasculaire chirurgie wordt met name toegeschreven aan de CPB. Echter, ook het transfunderen van rode bloedcellen zou kunnen bijdragen aan verhoogde vHb spiegels in deze setting. Opslag van rode bloedcellen leidt namelijk, zij het in beperkte mate, tot schade aan rode bloedcellen en zelfs tot hemolyse. Opslag van rode bloedcellen leidt om deze reden tot meetbaar verhoogde vHb concentraties in het opslagmedium. Tijdens de bloedtransfusie wordt gelijktijdig ook het vHb-bevattende opslagmedium getransfundeerd, hetgeen theoretisch een toename van vHb spiegels in het bloed van de ontvanger kan veroorzaken. Om dit te onderzoeken hebben wij 30 patiënten met een vorm van bloedkanker onderzocht die werden getransfundeerd met 1 of 2 opgeslagen rode bloedcelproducten (packed cells, **Hoofdstuk 8**). Wij hebben gekozen voor het bestuderen van dergelijke patiënten omdat in deze setting het hemolyserende effect van de CPB (zoals bij hart- en vaatchirurgie patiënten) geen rol speelt. Transfusie van 2 packed cells resulteerde in een statistisch significante toename van het vHb en de NO-consumptie in het bloed. Hoewel het opslagmedium van langer opgeslagen bloedproducten meer vHb bevatte en meer NO kon consumeren, resulteerde de transfusie van oudere packed cells niet tot hogere vHb spiegels of sterkere NO-consumptie in het bloed van de ontvanger in vergelijking met transfusie van kort opgeslagen packed cells. Daarentegen bleken patiënten met lage haptoglobine spiegels (het eiwit dat onder fysiologische omstandigheden vHb wegvangt) vòòr de transfusie, hogere vHb concentraties na de transfusie te vertonen. Omgekeerd lieten de patiënten met de hoogste pre-transfusie haptoglobine waarden geen stijging van het vHb zien, ongeacht of zij 1 of 2 packed cells ontvingen. Extrapolatie van deze resultaten naar de setting van (complexe) cardiovasculaire chirurgie zou kunnen betekenen dat transfusie van packed cells, vaak nodig om bijvoorbeeld bloedverlies tijdens de operatie te compenseren, de toename van vHb en NO-consumptie bij deze patiënten verder versterkt. Zeker indien uitgebreide transfusie noodzakelijk is, en patiënten lage haptoglobine spiegels hebben voor de operatie, kan de toename van vHb en NO-consumptie klinisch relevant worden; dat wil zeggen, potentieel bijdragen aan het ontstaan van darmschade, nierschade en postoperatieve nierfunctiestoornissen.

Gezien de bovenstaande resultaten achten wij het waarschijnlijk dat hemolyse een belangrijke, en beïnvloedbare, risicofactor is voor het ontstaan van viscerale schade tijdens en na (complexe) cardiovasculaire chirurgie. Het voorkomen of behandelen van

hemolyse zou dus kunnen leiden tot minder complicaties en een betere overleving van de patiënt. Hoewel de oorzaak van hemolyse moeilijk aan te pakken is, zijn er wel diverse veelbelovende interventies die de schadelijke effecten van vHb kunnen verminderen. In **Hoofdstuk 4** worden enkele voorbeelden van dergelijke interventies besproken die specifiek in deze setting van nut kunnen zijn. Een niet in dit hoofdstuk besproken therapeutische interventie hebben wij in **Hoofdstuk 9** onderzocht. In een hemolyse proefdiermodel, waarbij aan ratten vHb werd toegediend tot dat vergelijkbare concentraties als die in onze patiënten meetbaar waren, hebben wij onderzocht of het geven van vetrijke voeding beschermend werkt op het ontstaan van viscerale schade. Het aanbieden van voedsel in de darm leidt over het algemeen tot een verbeterde darm doorbloeding, onafhankelijk van NO. Bovendien heeft eerder onderzoek aangetoond dat vetrijke voeding in het bijzonder de lokale (in de darm) en systemische ontstekingsreacties vermindert. Vetrijke voeding stimuleert tevens de afgifte van het stofje acetylcholine door een belangrijke zenuw in ons lichaam; de nervus vagus. Acetylcholine kan, vergelijkbaar met NO, vaatverwijding veroorzaken. Het toedienen van vetrijke voeding voor de toediening van vHb resulteerde inderdaad in significant minder schade aan de darm, nier, en lever. Bovendien verbeterde de doorbloeding in deze organen significant vergeleken met dieren die geen vetrijke voeding gehad hadden. Hoewel we de rol van acetylcholine niet direct hebben kunnen aantonen was het beschermende effect afhankelijk van de nervus vagus.

Samenvattend laten de resultaten van dit proefschrift zien dat het optreden van hemolyse tijdens complexe cardiovasculaire chirurgie een klinisch relevant fenomeen is. Hemolyse leidt, via het wegvangen van NO door vHb, tot duidelijk verminderde concentraties NO in het bloed. Hierdoor is er minder NO beschikbaar om vaatverwijding in stand te houden, dan wel te stimuleren, wat resulteert in een verminderde doorbloeding van weefsels en organen. De mate van hemolyse en plasma NO-consumptie is significant gecorreleerd aan darm- en nierschade, en patiënten met hoge vHb waarden tijdens de operatie hadden een groter risico op postoperatieve nierfunctieproblemen in vergelijking met patiënten met lagere vHb concentraties. Hoewel de CPB de voornaamste veroorzaker van hemolyse is, kan het geven van bloedtransfusies de concentratie vHb in het bloed, en daarmee de NO-consumptie, nog verder laten toenemen. Lage haptoglobine concentraties, waardoor vHb minder goed weggevangen kan worden, kunnen dit effect nog verder versterken. Patiënten met bijvoorbeeld een reeds preoperatief verminderde functie van de nier hebben, met lage preoperatieve haptoglobine spiegels, die grote operaties ondergaan en die vele bloedtransfusies nodig hebben zijn potentieel het meest gevoelig voor de schadelijke effecten van hemolyse in deze setting.

Gezien deze resultaten beschouwen wij vHb als een potentieel waardevol therapeutisch doel om orgaanschade tijdens (complexe) cardiovasculaire operaties te verminderen. Het zal in de toekomst moeten blijken of dergelijke interventiestudies leiden tot een vermindering van postoperatieve complicaties en een verbeterde overleving van de patiënt.

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DANKWOORD

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Curriculum vitae

CURRICULUM VITAE

Iris Vermeulen Windsant werd op 19 november 1981 geboren in Haarlem, Nederland. Tussen 1994 en 2001 doorliep zij haar middelbare school aan de Rudolf Steiner School in Haarlem (1994-2000) en de Geert Groote School in Amsterdam (2001). Nadat zij haar VWO eindexamen (cum laude) behaalde begon zij in September 2001 met de studie Geneeskunde aan de Faculteit der Geneeskunde van de Universiteit Maastricht (later Faculty of Health Medicine and Life Sciences, Maastricht University). De eerste wetenschappelijke stappen werden in het derde studiejaar gezet door deelname aan onderzoek binnen de vakgroep Algemene Heelkunde (2003) bij Dr. Nicole Bouvy. Tijdens een keuze co-schap Cardiothoracale Chirurgie in 2004 verdiepte Iris zich verder in de wereld van het wetenschappelijk onderzoek onder begeleiding van Dr. Randolph van Eps. In het laatste jaar van haar studie (2006-2007) volgde tenslotte een keuze co-schap Vaatchirurgie onder leiding van Prof. dr. Geert Willem Schurink, afgewisseld door de wetenschapsstage bij de onderzoeksafdeling van de Algemene Heelkunde onder leiding van Prof. dr. Wim Buurman. Tijdens dat jaar werd het fundament gelegd voor een verdere wetenschappelijke vorming en verdieping. Enkele maanden voor het behalen van haar artsenbul in september 2007 (cum laude), startte Iris op 1 maart 2007, onder leiding van Prof. dr. Wim Buurman en Prof. dr. Michael Jacobs met het promotietraject waarvan dit proefschrift het resultaat is.

Van 1 oktober 2011 tot en met 31 januari 2012 werkte Iris als Anios Cardiologie in het Catharina Ziekenhuis in Eindhoven waar zij vervolgens op 1 februari 2012 met veel enthousiasme gestart is met haar medische specialisatie tot cardioloog onder leiding van Dr. Jan Melle van Dantzig (opleider) en Prof. dr. Nico Pijls (plaatsvervangend opleider).

Publicatielijst

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Abbreviations

ABBREVIATIONS

A

AAA - abdominal aortic aneurysm
ACC - aortic cross clamp
ACE - angiotensin converting enzyme
Ach - acetylcholine
ALT - alanine transaminase
AKI - acute kidney injury
AKIN - acute kidney injury network
ANOVA - analysis of variance
Arg - arginine
AST - aspartate aminotransferase
AUC - area under the curve

B

BT - blood transfusion

C

CABG - coronary artery bypass grafting
CABG + Valve - coronary artery bypass grafting + valve reconstruction (any type)
CCK-1R - cholecystokinin 1 receptor
CO - carbon monoxide
COPD - chronic obstructive pulmonary disease
CPB - cardiopulmonary bypass

D

DAP - distal aortic perfusion
DeoxyfHb - deoxygenated fHb [HbFe^{2+}]

E

ECC - extracorporeal circulation
ECMO - extracorporeal membrane oxygenation
ELISA - enzyme linked immunosorbent assay
en% - energy percent
ET - endothelin
EVAR - endovascular aortic repair

F

fHb - free hemoglobin
FBF - forearm blood flow

G

GFR - glomerular filtration ratio

H

HO - heme oxygenase
Hp - haptoglobin
HRP - horseradish peroxidase

I

ICAM - intercellular adhesion molecule
ICU - intensive care unit
IFABP - intestinal fatty acid binding protein
IL - interleukin
IN - intestinal necrosis
IQR - inter quartile range

Iron-nitrosylHb - iron-nitrosylhemoglobin
[$\text{HbFe}^{2+}\text{NO}$]

L

LFABP - liver fatty acid binding protein

M

MetHb - methemoglobin [HbFe^{3+}]
MDRD - modification of diet in renal disease
[formula]
MOF - multiple organ failure

N

nAChR - nicotinic acetylcholine receptor
NAG - N-acetyl- β -D-glucosaminidase
NO - nitric oxide
 NO_3^- - nitrate
(e)NOS - (endothelial) nitric oxide synthase
NS - non significant

O

Orn - ornithine
OPCAB - off-pump coronary artery bypass
[grafting]
OR-AAA - open repair of AAA
OR-TAA(A) - open repair of TAA(A)
OxyfHb - oxygenated fHb [$\text{HbFe}^{2+}\text{O}_2$]

P

PAOD - peripheral arterial occlusive disease
PEG - pegylated
PPM - parts per million
pRBC(s) - packed red blood cell(s)

R

RBC(s) - red blood cell(s)
Rh - rhesus
ROC - receiver operating characteristic
 R_s - spearman correlation

S

S.E.M. - standard error of the mean
SIRS - systemic inflammatory response
syndrome
SM - storage medium
SNP - sodium nitroprusside
SP - selective organ perfusion

T

TAA - thoracic aortic aneurysm
TAAA - thoracoabdominal aortic aneurysm

V

VCAM - vascular cell adhesion molecule

W

wt% - weight percent