Nitric oxide

An ally in extracorporeal circulation?

VILYAM MELKI
Many complications associated with heart surgery are due to the negative effects of extracorporeal circulation (ECC). Some of these complications may be attributed to ECC-induced activation of inflammation and coagulation pathways. The inflammatory reaction may be caused by the interaction of blood components with air and the artificial surfaces of the ECC, from substances produced due to ischaemia-reperfusion injury of the heart and lungs, and from increased release of endotoxin from ischemic intestines. Staphylococcus aureus (S. aureus) infections are the leading cause of respiratory, skin and soft tissue, and bloodstream infections. Nitric oxide (NO) is a gaseous signaling molecule involved in many physiological and pathological processes. The role of NO in infection and inflammation is complex. NO may contribute to morbidity by acting as a vasodilator, myocardial depressant, and cytotoxic mediator. On the other hand, NO may have a salutary role through microvascular, cytoprotective, immunoregulatory, and antimicrobial properties. A simulated extracorporeal circulation (SECC) model is a closed circuit, including a roller pump, an oxygenator, a venous reservoir and polyvinyl chloride (PVC) tubing, where human blood is circulated. The SECC model allows studies of the blood and its components, without any influence from other organ systems. The aim of this work was to investigate NO effects during SECC and in S. aureus infection.

**Study I.** Human blood was circulated through SECC during 3 hours, and leukocyte granule release was studied. Results indicated that NO addition during SECC is pro-inflammatory by stimulating leukocyte activation and granule release, and has no effect on oxygen free radical production and interleukin release.

**Study II.** Investigating the effect of NO on S. aureus growth in whole blood during 180 min SECC, results showed a 6.2 fold growth in the presence of NO. Results indicated that by stimulating the expression of inducible lactate dehydrogenase, specific to S. aureus, NO may improve S. aureus resistance to oxidative stress, giving the pathogen a survival advantage.

**Study III.** In an in vitro system of SECC, we measured glyceryl trinitrate (GTN) induced changes in leukocyte activation in whole blood caused by S. aureus infestation, as well as the effect of GTN on S. aureus growth. Results indicated that GTN does not affect S. aureus growth during SECC and has no effect on SECC-induced leukocyte activation.

**Study IV.** Whole blood concentrations of selected leukocyte adhesion molecules, complement system components and myeloperoxidase were measured in an in vitro system of SECC. Results indicated that SECC induces the increased expression of some leukocyte markers and that GTN addition significantly reduces the expression of some leukocyte activation markers.

**Keywords:** Simulated extracorporeal circulation, Inflammation, Nitric oxide, Leukocyte activation, Staphylococcus aureus, Glyceryl trinitrate, Postoperative infection

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To my family, for your endless love and support
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

ACT  Activated clotting time
ADP  Adenosine diphosphate
AUC  Area under curve
C3a  Activated complement factor 3
C5a  Activated complement factor 5
Ca2+ Ionized calcium
CD   Clusters of differentiation
CFU  Colony-forming units
CL   Chemiluminescence
cNOS Constitutive nitric oxide synthase
CPD  Citrate-phosphate dextrose
CPM  Counts per minute
Cu/Zn Copper/Zinc
DNA  Deoxyribonucleic acid
ECC  Extracorporeal circulation
EDTA Ethylene-diamine-tetra-acetic acid
ELISA Enzyme-linked immunosorbent assay
FAD  Flavin-adenine-dinucleotid
FITC Fluorescein isothiocyanate
FSC/SSC Forward scatter/side scatter
GTN  Glyceryl-tri-nitrate
Hb   Hemoglobin
HLA-DR Human leukocyte antigen-DR
HNL  Human neutrophil lipocalin
HOCl Hypochlorous acid
HSA  Human Serum Albumin
IgA  Immunoglobulin A
IgG1 Immunoglobulin G1
IgG2 Immunoglobulin G2
IL-1β Interleukin-1-beta
IFN-γ Interferon-gamma
iNOS Inducible nitric oxide synthase
IR   Ischaemia-reperfusion
IU   International unit
kPa  Kilopascal
LF   Lactoferrin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LMW</td>
<td>Low molecule weight</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mab</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>N2</td>
<td>Nitrogen gas</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide-adenine-dinucleotide-phosphate</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>Ammonium Chloride</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO•</td>
<td>Nitric oxide radical</td>
</tr>
<tr>
<td>NO2</td>
<td>Nitrogen dioxide</td>
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<tr>
<td>NO2•</td>
<td>Nitrogen dioxide radical</td>
</tr>
<tr>
<td>NO3</td>
<td>Nitrate</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>O2-</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCO2</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>pH</td>
<td>The decimal logarithm of the reciprocal of the hydrogen ion activity</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PO2</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PPM</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RSNO</td>
<td>S-nitrosothiol</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SECC</td>
<td>Simulated extracorporeal circulation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIR</td>
<td>Systemic inflammatory response</td>
</tr>
<tr>
<td>SOZ</td>
<td>Serum opsonized zymosan</td>
</tr>
<tr>
<td>SSI</td>
<td>Surgical site infection</td>
</tr>
<tr>
<td>SST</td>
<td>Serum separating tube</td>
</tr>
<tr>
<td>TCC</td>
<td>Terminal complement complex</td>
</tr>
<tr>
<td>TNF- α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells count</td>
</tr>
<tr>
<td>WBL</td>
<td>Whole blood</td>
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1. Introduction

Before the mid-50’s, heart surgery was more of a curiosity, with poor patient outcome. With the introduction of extracorporeal circulation (ECC), surgeons were able to perform advanced heart operations with high accuracy and excellent outcome, helping millions of patients suffering from atherosclerotic coronary artery disease, congenital heart defects, heart valve disorders and aortic aneurysms. ECC soon became a technique used not only in heart surgery but also in dialysis and extracorporeal membrane oxygenation [1].

Despite the vast knowledge and impressing development of cardiovascular technologies, there are still considerable risks for complications related to heart surgery. Many of the complications associated with heart surgery are partly due to the negative effects of ECC itself, and some of these complications may be directly attributed to ECC-induced activation of inflammation and coagulation pathways.

During ECC, the blood is in direct contact with artificial surfaces, which subsequently may result in a systemic inflammatory response (SIR) similar to sepsis [2-4].

Staphylococcus aureus (S. aureus) is a major pathogen contributing to the much feared infectious complications after heart surgery. The usage of antibiotics as prophylaxis in heart surgery has been beneficial in the prevention of wound infections, but the development of antibiotic resistance among pathogens is alarming [5].

Nitric oxide (NO) is a gaseous paracrine and autocrine signaling molecule involved in many physiological and pathological processes, including inflammation and infection. NO-related antimicrobial activity has been demonstrated against a broad range of pathogenic microorganisms, but the role of NO in infection and inflammation is complex [6,7].

A simulated extracorporeal circulation (SECC) model is a closed circuit that allows studies of the blood and its components, without any influence from other organ systems.

The focus of this thesis was to investigate the role of NO in events occurring during SECC.
2. Background

2.1. Extracorporeal circulation

ECC is a technique where the function of the heart and the lungs has been replaced by a mechanical pump and a gas-exchanging oxygenator (heart-lung machine). The heart-lung machine is connected to the patient’s bloodstream via polyvinyl chloride (PVC) tubings inserted into the patient’s vascular system. To prevent blood clotting, the patient is perioperatively administered full-dose heparin, keeping an activated clotting time (ACT) > 400 seconds during ECC.

*Figure 1.* Schematic principles of extracorporeal circulation.
The ECC circuit is an open circuit where deoxygenated blood from the body, through a venous cannulae inserted usually into the right atrium, and blood collected from the operating field, through cardiotomy suction, drains into a venous reservoir. After passing through an oxygenator, supplying the blood with oxygen, an arterial filter removes any air bubbles, and a roller/centrifugal pump infuses the oxygenated blood back into the body usually via the aorta (Figure 1). A heat exchanger connected to the circuit allows control of the blood/body temperature [4].

2.2. Simulated extracorporeal circulation

The SECC model includes a barely occlusive roller pump, a hollow fiber oxygenator, a venous reservoir and PVC tubing. Human blood, drawn from healthy donors and heparinized, enters the circuit by gravity, without foaming, and is circulated. The temperature of the blood in the circuit is regulated by a thermostatic heat exchanger (Figure 2).

Figure 2. Schematic principles of simulated extracorporeal circulation.
2.3. Extracorporeal circulation and inflammation

During ECC, four plasma protein systems are activated; kallikrein, complement, coagulation and fibrinolysis. Also cellular elements within the blood stream are primed; platelets, granulocytes, monocytes and lymphocytes. Priming means that the cells are ready to perform an early-immediate response when they are challenged by another activation stimulus. After returning into the patient’s own vascular bed, blood that has been primed in the ECC activates, in turn, the patient’s own endothelial lining. Leukocyte-endothelial cell interactions, with cell activation leading to excretion of several active substances from cellular granulae, production of free cytokines, oxygen radicals, leukotrienes and prostaglandins etc, eliciting inflammatory reactions within the interstitial space of many tissues, with the heart and the lungs being the first and the most gravely affected target organs [2,3].

The inflammatory reaction is caused not only by the interaction of blood components with air and the artificial surfaces of the ECC, but also from substances produced due to ischaemia-reperfusion injury (IR) of the heart and lungs, and from increased release of endotoxin from ischemic intestines. IR is characterized by the generation of reactive oxygen species (ROS), elevation of intracellular calcium concentrations, inflammatory reaction, and sometimes can lead to cell death. IR can cause thrombin deposition which triggers inflammatory cells to release cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and the expression of adhesion molecules. The inflammation is further driven by the physical trauma of surgery [4].

The predominant cell type involved in the inflammatory response after ECC, are neutrophils. Activation of neutrophils may occur as a reaction to heparin-protamin or in response to complement activation. Complement factor 5a (C5a) can induce neutrophil aggregation and margination, and the binding of C5a to specific neutrophil membrane receptors stimulates neutrophil adherence to vascular endothelium. The complement fractions C3b and inactivated C3b, bound to the materials of the extracorporeal circuit, may induce neutrophil degranulation and monocyte release of interleukin-1β (IL-1β). Additional terminal complement cleavage products (C5b-9) generated during cardiopulmonary bypass could augment neutrophil activation by stimulation of intracellular arachidonic acid metabolism.

Being important markers of inflammation, some cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6, are known to be raised following cardiopulmonary bypass. They serve for signal communication between cells involved in immunity and inflammation and are thought to play an essential role in the pathogenesis of shock and multiple organ failure during sepsis [3].

Myeloperoxidase (MPO), human neutrophil lipocalin (HNL) and lactoferrin (LF) are proteins which are stored in neutrophil granulocytes and secret-
ed upon activation of the cells. These proteins are known to be markers of neutrophil presence and activity in circulating blood. Measurement of these soluble markers in biological fluids has been proposed to mirror the degree of neutrophil activity [8-10] (Figure 3).

![Figure 3. Inflammatory reactions related to extracorporeal circulation.]

**Myeloperoxidase**

MPO is a peroxidase enzyme, most abundantly expressed in neutrophil granulocytes and monocyte lysosomes, characterized by powerful pro-oxidative and proinflammatory properties. MPO catalyzes the conversion of chloride and hydrogen peroxide to hypochlorite and hypochlorous acid (HOCl) during the neutrophil’s respiratory burst. In addition, MPO consumes endothelial-derived nitric oxide (NO), thereby reducing NO bioavailability and impairing its vasodilating and anti-inflammatory properties. MPO also oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as an oxidizing agent. Although phagocytes have other microbicidal mechanisms, including release of antimicrobial peptides (e.g. defensins) and broadly acting proteases, phagocytosis with generation of ROS and HOCl is still regarded as the critical killing mechanism for most invading pathogens, including MPO-chloride-mediated killing of tumour cells, inactivation of chemotactic factors, and cross-linking of proteins [11,12].

**Human neutrophil lipocalin**

HNL is a protein from human neutrophil secondary granules. Its concentration increases in the sera of patients with acute bacterial infections, which is consistent with HNL’s proposed function as an endogenous bacteriostatic protein [13,14]. Most lipocalins are transport molecules that bind and transport small lipophilic substances. HNL is expressed and secreted by immune cells, hepatocytes, and renal tubular cells in various pathologic states. Bacteria produce siderophores (small iron-binding molecules) to scavenge iron from the extracellular space, ensuring their iron supply. Siderophores are the strongest iron chelators known and are able to extract iron from most other organic complexes, in particular, from other iron-binding proteins such
as lactoferrin and transferrin. HNL exerts bacteriostatic effects, by its ability to capture and deplete siderophores. It is thereby a potent bacteriostatic agent in iron-limiting conditions [14].

Growing evidence suggests effects of HNL beyond fighting microorganisms. HNL acts as a modulator of various cellular responses, such as proliferation, apoptosis, and differentiation [15].

**Lactoferrin**
LF is an iron-binding glycoprotein of the transferrin family, which is stored in the secondary granules of neutrophils. LF is considered a first-line defense protein involved in protection against a multitude of microbial infections and prevention of systemic inflammation, and it has been implicated in protection against cancer by its various immunomodulatory properties and its ability to promote apoptosis in cancer cells. LF has a profound modulatory action on the adaptive immune system by promoting the maturation of T-cell precursors into competent helper cells and by the differentiation of immature B-cells into efficient antigen presenting cells. In addition, LF augments the delayed type hypersensitivity response to antigens, leading to a strong induction of cell-mediated immunity in mice. LF has bacteriostatic and bactericidal effects, the former being a result of iron sequestration by LF and the latter dealing with LF capabilities to bind lipopolysaccharide (LPS). The ability of LF to bind large quantities of iron also provides protection against pathogens and their metabolites by stimulating phagocytosis and cell adherence and controlling the release of proinflammatory cytokines. For example, LF modulates cytokine release from host cells, enhancing IL-6 and TNF-α release from peripheral blood mononuclear cells but inhibiting release of IL-1 and IL-2. With its iron binding property, LF exerts its multiple antibacterial effects by depriving microorganisms of this crucial nutrient and limiting their growth. It has been suggested that LF also possesses antibacterial activity, independent of iron chelation, by binding to the LPS layer of bacterial cell wall which causes increased membrane permeability and bacterial cell lysis [16-19].

**Interleukin-1-beta**
IL-1 is produced mainly by cells of the innate immune system. IL-1 increases the expression of adhesion molecules on endothelial cells thereby enabling transmigration of immunocompetent cells, such as phagocytes, lymphocytes and others, to sites of infection. IL-1β is one of the most studied proinflammatory cytokines [20]. As prominent and early mediators of inflammation, pro-inflammatory cytokines critically regulate the response to cardiac injury. IL-1 signaling mediates chemokine synthesis in the infarcted myocardium and stimulates infiltration of the infarct tissue with the leukocytes. Neutrophils are recruited very early after cardiac injury, pro-inflammatory monocytes and lymphocytes follow. Leukocyte transmigration
in the infarcted myocardium requires adhesive interactions with activated vascular endothelial cells. First, leukocytes are “captured” from the rapidly flowing bloodstream and roll on vascular endothelial cells through interactions involving a family of cell adhesion receptors, the selectins. The next step is stable arrest, characterized by firm adhesion of the leukocyte on the endothelial surface mediated through chemokine-induced activation of leukocyte integrins that bind to their endothelial counter receptors (such as Vascular Cell Adhesion Molecule-1, Intercellular Adhesion Molecules 1 and 2, and the members of the Junctional Adhesion Molecules family). Transendothelial migration follows at both junctional and non-junctional locations leading to infiltration of the infarcted tissue with leukocytes [21].

Interleukin-10
Interleukin-10 (IL-10) is an anti-inflammatory cytokine with pleiotropic effects in immunoregulation and inflammation. IL-10 is predominantly expressed by stimulated monocytes and activated T lymphocytes, and prevents excessive inflammatory reactions.

Inflammatory responses are dependent on activation of adhesive interactions between endothelial cells and leukocytes. IL-10 suppresses adhesion molecule expression, down modulating adhesive interactions. IL-10 inhibits the production of pro-inflammatory cytokines and chemokines by endotoxin-stimulated macrophages and regulatory T-cells such as interferon-gamma (IFN-γ), IL-2 and TNF-α, suppressing the inflammatory response. IL-10 induction in the infarcted heart may play a role in controlling the acute inflammatory response. Corticosteroid pretreatment of patients undergoing cardiac surgery with ECC, increases blood IL-10 levels and prevents proinflammatory cytokine release, resulting in better heart and lung protection after ECC [2,21,22].

Terminal Complement Complex (TCC) and activated complement factor 3 (C3a)
The complement system is a danger sensing system, consisting of a number of small proteins normally circulating in the blood as inactive precursors. The complement system recognizes exogenous threats such as conserved microbial motifs as well as endogenous threats including altered-self molecules (e.g. following injury or hypoxia, after virus-infection or tumor-related) and apoptotic cells [23]. The basic functions of complement are opsonization, chemotaxis, cell lysis and clumping of antigen-bearing agents. The complement system is activated in three ways: via the classical pathway, which includes the proteins C1, C4, C2, and C3; the alternative pathway, with the participation of C3 and protein factors B, D, and P; and the lectin pathway, with the participation of mannose-binding lectin (MBL) and MBL-associated proteases. All three pathways lead to the activation of the C5, C6, C7, C8, and C9 proteins, and intermediate formation of C5b-7, C5b-8, and
C5b-9 (Figure 4). These complexes are collectively referred to as TCCs, while C5b-9, the final complex and the most effective at inducing cell death, is referred to as the cell-killing membrane attack complex [24,25].

Danger sensing molecules that activate the complement system comprise soluble C-type lectins such as MBL, ficolins, the C-type lectin-like molecule C1q and properdin. Once bound to danger motifs, they initiate activation of proteolytic cascades that result in the cleavage of the central molecule of the complement system, C3, followed by cleavage of C5. Together with other cleavage products of C3, the smaller cleavage products of C3 and C5, C3a and C5a, form a set of soluble mediators that bind distinct cell surface receptors expressed on a variety of target cells. The interaction of C3a and C5a with their cognate receptors induces pleiotropic effector functions, translating the danger information from the fluid phase into defined cellular responses. C3a and C5a regulate vasodilation, increase the permeability of small blood vessels, and induce contraction of smooth muscles. In macrophages, neutrophils, and eosinophils C3a and C5a can trigger oxidative burst [23,24].

Figure 4. Main functions of complement: recruitment of inflammatory cells, opsonization and killing of pathogens.
2.4. Systemic inflammatory response

Almost all surgical or trauma patients develop a mild subclinical form of SIR which helps the patient to overcome the temporary loss of homeostasis inflicted by trauma, surgery or infection. This biological response is a prerequisite for regaining the regulatory homeostatic mechanisms and for proper injury healing on all organ levels. Since there are no optimal biocompatible materials, the blood in the heart-lung machine is in direct contact with artificial surfaces, resulting in activation of various blood components and, in its most extreme form, a subsequent SIR similar to sepsis.

Attempts have been made to reduce the incidence of SIR and its complications through drug administration, limiting the blood-air interface, decreasing the surface area of artificial material, and optimizing the surface coating of components [2-4,8].

When evading the network of regulatory mechanisms, SIR leads to several adverse clinical phenomena. Coagulopathy, increased vascular leakage of fluid, peripheral circulatory disorders, tachycardia, tachypnea and fever are some of most frequent ones. This may significantly derange the haemodynamic stability of patients even for long period after the cessation of ECC, increasing the time required for recovery on the intensive care unit.

The clinical consequences ranges from barely discoverable functional disorders to reversible more or less extensive injuries of brain, heart, lungs, kidneys and other organs, which may progress to multiorgan failure or irreversible organ damage. This contributes to the morbidity and mortality associated with cardiac surgery.

In the initial stages of the critical illness, SIR is characterized by predominance of pro-inflammatory mediators, such as TNF-α and/or IL-1β. If successfully treated, these gravely affected patients enter an intermediate state with both pro- and anti-inflammatory mediators present in nearly equal amounts. As the patient’s clinical status and overall prognosis is improving, the anti-inflammatory mediators are predominating [2-4,9].

2.5. Leukocytes

All leukocytes derive from a multipotent hematopoietic stem cell. Leukocytes are divided into five classes: neutrophils, eosinophils, basophils, monocytes, lymphocytes.

Collectively, neutrophils, eosinophils, and basophils are known as granulocytes due to the presence of granules in their cytoplasm. In addition, monocytes and lymphocytes are also known as mononuclear cells [26].
Neutrophils
Neutrophils are usually first responders to microbial infection. Neutrophils circulate in blood as quiescent cells that are recruited to sites of inflammation, primarily by local changes in the endothelial cells, which activate the neutrophils to become adherent to the endothelium and migrate out into tissues to engulf microorganisms by phagocytosis and then release enzymes (such as lysozyme) to destroy the infectious pathogens [27].

Monocytes
Monocytes are larger than other leukocytes, and they mature into macrophages when they have migrated from the bloodstream into the body’s organs and tissues. Like neutrophils, monocytes are phagocytic but they live longer and they have an additional role: they present pieces of pathogens to T cells so that the pathogens may be recognized again and killed, or so that an antibody response may be mounted.

Lymphocytes
Three types of lymphocytes are found in the blood; B-cells, characterized by the presence of immunoglobulins on their surface, and upon stimulation with antigen, transformed into antibody producing plasma cells. T-cells, who take part in cell mediated immune response, which does not depend on the presence of circulating antibodies. The T cells coordinate the immune response and are important in the defense against intracellular bacteria. Natural killer cells are able to kill cells of the body which are displaying a signal to kill them, as they have been infected by a virus or have become cancerous [26].

2.6. Cluster of differentiation (CD)
CD molecules are cell surface markers useful for the identification and characterization (immunophenotyping) of leukocytes. These markers are often used to associate cells with certain immune functions. Physiologically, CD molecules can act in numerous ways, acting as receptors or ligands, or in cell adhesion [28]. The proposed surface molecule is assigned a CD number once two specific monoclonal antibodies are shown to bind to the molecule [29].

2.7. Cardiac surgery and infection
Infections are among the worst clinical complications after cardiac surgery, with a reported incidence of 2-4%. Surgical site infections (SSI) after cardiac operations are associated with severe outcomes, including increases in hospi-
tal length of stay, readmission rates, health care costs, and high morbidity rates.

Antibiotic prophylaxis in cardiothoracic surgery have demonstrated a significant and large benefit in the prevention of wound infections. Antibiotic prophylaxis seems though to affect mainly SSIs caused by Gram positive bacteria, while those SSIs caused by Gram negative bacteria are less affected by the type of prophylaxis regimen. SSIs incur significant morbidity and costs, but rarely lead to death [30].

By decolonizing patients who carry S. aureus and potentially by adding a glycopeptide to the usual prophylaxis using β-lactam antibiotics for methicillin-resistant S. aureus carriers, SSIs, caused by Gram positive bacteria, may be prevented [31].

2.8. Staphylococcus aureus

S. aureus is a major pathogen that colonizes skin and mucous membranes, with the anterior nares being the primary niche in humans, and it is found in 30-50% of the healthy human population. S. aureus infections are the leading cause of respiratory, skin and soft tissue, and bloodstream infections. The majority of S. aureus strains have developed drug-resistance against every antibiotic licensed for the therapy of staphylococcal infections. Of particular concern is the emergence of methicillin-resistance and acquisition of additional antibiotic resistance including vancomycin. Hygienic measures reduce the burden of staphylococcal infections.

The S. aureus genome is mainly composed of a backbone of genes that are conserved among the different strains. S. aureus also has an ‘accessory genome’, which is variable between strains. This ‘accessory genome’ largely contributes to the high genetic and phenotypic plasticity of the pathogen. S. aureus uses a wide variety of immune escaping systems and virulence factors [5].

Neutrophils are the primary host defense against staphylococcal infection [32]. S. aureus possesses a variety of vigorous molecules and mechanisms to combat and diminish neutrophil defenses including secretion of proteins that inhibit the deposition and activation of complement; the secreted staphylococcal complement inhibitor protein interacts directly with both C3 convertases and stabilizes them, resulting in inhibition of further C3b deposition, and Staphylococci can also interfere directly with the products of the C5 convertases.

S. aureus generates molecules that prevent or deter neutrophil recruitment to the sites of infection; superantigen-like protein 7 strongly inhibits C5a neutrophil recruitment through IgA dependent binding to C5, preventing its cleavage, and the chemotaxis inhibitory protein interacts directly with the C5a receptor on neutrophils blocking C5a chemoattractant signaling.
S. aureus strains also express immunoglobulin binding molecules. The interaction with and sequestration of anti-staphylococcal immunoglobulins mediated by these proteins may severely limit host immunoglobulin mediated immune clearance of the pathogen [33] (Figure 5).

Figure 5. Summary of S. aureus immune evading systems and virulence factors.

Several bacterial factors also mediate interactions with phagocytes, aiding survival within the phagocyte. S. aureus is capable of secreting cytolytic toxins which as well as killing the phagocyte and liberating key nutrients, such as heme bound iron, for extracellular staphylococci, may provide intracellular staphylococci an escape from phagolysosomes. In addition to secreted factors to interfere with phagocytic cells, S. aureus also retains surface associated virulence factors that inhibit phagocytic uptake.

Nutritional immunity (the sequestration of essential metals, such as iron, manganese and zinc) is a potent defense against invading pathogens such as S. aureus [34].

The human host has a number of strategies to limit iron availability to invading pathogens such as S. aureus. During inflammation, there is increased levels of the peptide hormone hepcidin. Hepcidin suppress release of iron by macrophages and by duodenal enterocytes, concomitant with increased synthesis of macrophage ferritin. At the site of infection, additional iron withholding defenses are mediated by professional phagocytes, releasing iron binding transferrins and bacterial siderophore binding lipocalins. Other described defenses include hepatic release of haptoglobin and hemopexin to bind and scavenge extracellular hemoglobin (Hb) and heme and B lymphocyte production of immunoglobulins to microbial cell surface receptors that mediate pathogen iron uptake (Figure 6).
S. aureus have evolved diverse mechanisms to acquire iron and overcome the host defenses aimed at acquiring iron from the pathogen. These systems can be broadly divided into siderophore-mediated uptake mechanisms and heme transport mechanisms [33].

Host iron and iron binding proteins can also have secondary effects on inflammation and hemostasis. Hb liberated from red blood cells, lysed by bacterial hemolysins for example, can bind and inactivate NO. This results in contracted vessels and increased platelet aggregation, both of which contribute to thrombosis [35].

2.9. Nitric oxide

NO is a lipophilic free radical and a powerful vasodilator, biosynthesized endogenously from L-arginine, oxygen, and NADPH by various nitric oxide synthase (NOS) enzymes. Reduction of inorganic nitrate may also serve to produce NO. NO is highly reactive and diffuses freely across membranes [36].

The endothelium is important in regulating vascular homeostasis by elaborating factors such as angiotensin II, NO, endothelin, and prostaglandins. The net effect is maintenance of normal vascular tone. The endothelium also maintains normal blood viscosity, prevents abnormal blood clotting, and prevents abnormal bleeding in terms of a balance between plasminogen acti-
vator inhibitor-1 and tissue plasminogen activator. It limits inflammation of
the vasculature and it can suppress smooth muscle cell proliferation.

Abnormally functioning endothelial cells cause decreased NO formation
and a decrease in vasodilatation, as well as decreased angiotensin II and
prostaglandin formation. The net effect is increased inflammation and hyper-
trophy of the smooth muscle cells, promotion of thrombosis and vasocon-
striction, which creates a situation ripe for establishment and rapid growth of
atherosclerotic plaques [37].

The diverse list of biological processes that NO is associated with in-
cludes vessel homeostasis by NO inhibition of vascular smooth muscle con-
traction and growth, platelet aggregation, and leukocyte adhesion to the en-
dothelium, the immune response to infection where NO is generated by
phagocytes as part of the human immune response, wound repair, as well as
cancer biology and pathology. NO can contribute to reperfusion/ischemia
injury when reacting with superoxide to produce the damaging oxidant per-
oxynitrite [6,38-40].

Basal formation of NO varies considerably from one vessel type to anoth-
er and across differing diameters in the same vessel segment.

Shear forces trigger the opening of calcium channels on endothelial cells,
thereby leading to the calcium-dependent activation of endothelial NOS and
increase local production of NO (Figure 7). However, both calcium-
dependent and calcium- independent activation of endothelial NOS can oc-
cur and lead to NO- mediated vasorelaxation in response to shear stress.
The small size and lipophilic nature of NO are conducive to the rapid diffu-
sion of NO through cell membranes to reach its target cells. The chemically
labile property of NO allows for a truly local action, as does the high binding
affinity of erythrocyte for NO [41].

Two important biological reaction mechanisms of NO are S-nitrosation of
thiols, and nitrosylation of transition metal ions. S-nitrosation involves the
(reversible) conversion of thiol groups, including cysteine residues in pro-
teins, to form S-nitrosothiols (RSNOs) [42]. The second mechanism, nitro-
sylation, involves the binding of NO to a transition metal ion like iron or
copper. In this function, NO is referred to as a nitrosyl ligand. Typical cases
involve the nitrosylation of heme proteins like cytochromes, thereby disa-
bling the normal enzymatic activity of the enzyme. Hb is a prominent exam-
ple of a heme protein that may be modified by NO by both pathways: NO
may attach directly to the heme in the nitrosylation reaction, and inde-
pendently form RSNOs by S-nitrosation of the thiol moieties [7].

There are several mechanisms by which NO has been demonstrated to af-
fact the biology of living cells. These include oxidation of iron-containing
proteins such as ribonucleotide reductase and aconitase, activation of the
soluble guanylate cyclase, ADP ribosylation of proteins, protein sulfhydryl
group nitrosylation, and iron regulatory factor activation [43].
NO stimulates the soluble guanylate cyclase, which is a heterodimeric enzyme, with subsequent formation of cyclic guanylate-mono-phosphate and activation of protein kinase G, which causes reuptake of Ca2+ and the opening of calcium-activated potassium channels. The fall in concentration of Ca2+ ensures that the myosin light chain kinase can no longer phosphorylate the myosin molecule and thereby stopping the crossbridge cycle leading to relaxation of the smooth muscle cell [44].

*Figure 7. Summary of NO production endogenously and from reduction of glycercyltrinitrate (GTN), and mechanisms by which NO affects the biology of living cells.*
2.10. NO and infection

The role of NO in infection is complex, influencing it in various ways depending on existing circumstances. NO may contribute to morbidity by acting as a vasodilator, myocardial depressant, and cytotoxic mediator. On the other hand, NO may have a salutary role in the infected host through microvascular, cytoprotective, immunoregulatory, and antimicrobial properties [6,7].

NO-related antimicrobial activity has been demonstrated against a broad range of pathogenic microorganisms including viruses, bacteria, fungi, and parasites. NO production by the inducible NO synthase (iNOS) is stimulated by proinflammatory cytokines such as IFN-γ, TNF-α, IL-1, and IL-2, as well as by microbial products such as LPS. Infections in humans are often associated with significant increases in systemic NO production, determined by measurement of NO endoxidation products nitrite (NO₂⁻) and nitrate (NO₃⁻) in plasma and urine [45].

Also, abrogation of iNOS activity produces dramatic increases in microbial burden [46], and NO-donor compounds have been shown to inhibit or kill microbes when directly administered in vitro [47]. Latent infections require active suppression by host cellular immune mechanisms and studies have shown that NO may play a central role in persistent or latent infections [46].

High-output NO synthesis by iNOS seems to be part of the antimicrobial armamentarium of human macrophages [48].

Both iNOS and the phagocyte nicotinamide-adenine-dinucleotide-phosphate (NADPH) oxidase may be costimulated by inflammatory stimuli (e.g., IFN-γ). Simultaneous production of reactive nitrogen and oxygen intermediates may lead to the formation of a variety of antimicrobial molecular species. In addition to NO radical itself, potentially important NO congeners include peroxynitrite, RSNOs, nitrogen dioxide, dinitrogen trioxide, dinitrogen tetroxide, and dinitrosyl–iron complexes [45].

Interactions between reactive oxygen and nitrogen intermediates provide a molecular basis for synergy between the respiratory burst and synthesis of NO. Reaction products such as peroxynitrite can have greater cytotoxic potential than NO’ or O₂⁻ alone [49]. The combination of hydrogen peroxide and NO’ appears to possess particularly potent antibacterial activity [50].

In some circumstances though, the combination of reactive oxygen and nitrogen intermediates may be antagonistic; NO may protect mammalian cells against oxidant injury, perhaps by forming iron–nitrosyl complexes (making iron less available for catalysis of prooxidant reactions), inhibiting the respiratory burst oxidase, directly scavenging radical species, or inducing the expression of protective stress regulons. NO’ may also reduce oxidant membrane injury by terminating lipid peroxidation reactions. Thus, NO production may simultaneously enhance the antimicrobial function of the respirato-
ry burst while protecting tissues from oxidant injury. Interactions with \( \text{O}_2^- \)
can reduce concentrations of NO\(^\cdot\), which may diminish antimicrobial activity
toward microbes more sensitive to NO\(^\cdot\) than to peroxynitrite [45].

NO\(^\cdot\) itself does not possess antimicrobial activity for Escherichia coli
[50], but RSNOs are bacteriostatic and peroxynitrite is bactericidal for these
organisms. In contrast, RSNOs and NO\(^\cdot\) are microbicidal for Staphylococcus
aureus, Leishmania major, and Giardia lamblia, under conditions in which
peroxynitrite does not exert an apparent antimicrobial effect. Reactive nitro-
gen intermediates have been shown to modify deoxyribonucleic acid (DNA),
proteins, and lipids, as well as exert indirect effects on microbes by modulating
immune responses or other host cell functions [45].

DNA is an important target of reactive nitrogen intermediates. NO can
deaminate DNA in vitro [51]. Nitrogen dioxide radical (NO\(_2^\cdot\)) and peroxyni-
trite can also oxidatively damage DNA [52]. Some of the effects of reactive
nitrogen intermediates on DNA may involve interactions with DNA repair
systems, as well as direct modification of deoxyribonucleotides. NO interac-
tions with proteins can involve reactive thiols, heme groups, iron-sulfur clus-
ters, phenolic or aromatic amino acid residues, tyrosyl radicals, or amines.
Peroxynitrite and NO\(_2^-\) can also nonspecifically oxidize proteins at a variety
of sites [53].

NO\(^\cdot\) can interact with iron contained in heme proteins such as guanylyl
cyclase. Although guanylyl cyclase is activated by NO, NO–heme interac-
tions can result in the inactivation of other heme proteins, such as catalase
and cytochrome systems [54].

Low molecular weight (LMW) thiols have an important scavenging role
in microbes. High intracellular thiol concentration, such as glutathione, in
enteric bacteria may explain the lack of antimicrobial activity normally ex-
erted by NO\(^\cdot\) against these pathogens. In contrast, Staphylococci contain low
concentrations of glutathione [55], and appear to be susceptible to NO\(^\cdot\)[56].
Homocysteine is another LMW thiol compound which has been implicated
in resistance to RSNOs.

Microbial systems which repair oxidative injury appear to be similarly in-
volved in repairing nitrosative injury. Glucose-6-phosphate dehydrogenase,
which provides a major source of reducing equivalents (NADPH) used to
regenerate thiols and other antioxidants, is similarly involved in defenses
against both reactive oxygen and nitrogen intermediates. Cu/Zn superoxide
dismutase may protect against both oxidative and nitrosative stress by re-
moving periplasmic superoxide and limiting peroxynitrite formation.

In summary, reactive species derived from NO seem to possess biologi-
cally important antimicrobial activity. This is supported by studies showing
that: (a) increased NO production is associated with host defense in human
infections; (b) inhibition of inducible NO synthase enhances microbial replica-
cation. NO appears to be of particular importance in host defense against
intracellular pathogens, and perhaps in the maintenance of microbial latency.
NO may act in concert with ROS to damage microbial DNA, proteins, and lipids. Microbial defenses against oxidative and nitrosative stresses share many elements in common, including specific stress regulons, scavengers, detoxifying enzymes, repair systems, and strategies to subvert or avoid host phagocytes [45].

2.11. Vascular NO formation

NO formation by endothelial NOS (eNOS) plays an important role in the regulation of vasomotor tone in the pulmonary and systemic vascular beds. Once released from the endothelium, NO diffuses into vascular smooth muscle cells inducing vasodilatation and into the blood stream where it inhibits platelet aggregation and inflammation. Loss of endogenous NO activity has a number of detrimental consequences, most notably, vasoconstriction, increased activity and adherence of platelets, and accumulation of inflammatory cells at sites of endothelial damage. Endothelial damage is associated with most forms of cardiovascular disease, including hypertension, coronary artery disease, heart failure, peripheral artery disease, diabetes, chronic renal failure, and pulmonary hypertension (Figure 7).

2.12. Glyceryl-trinitrate (GTN)

Current indications for nitrate therapy include angina pectoris, complications of acute myocardial infarction, and ventricular unloading in heart failure [57]. In the perioperative period, nitrates are also used by anesthesiologists and intensivists to control systemic and pulmonary arterial pressure [57,58]. Endothelial function is altered in the presence of cardiovascular risk factors, suggesting that endothelial dysfunction is likely to be an important first step in CVD [59]. Endothelial dysfunction arises from down-regulation of endothelial NOS expression and activity, and uncoupling of NOS generating free radicals [60]. GTN seems to have a direct action on veins to reduce preload, whereas amyl nitrite reduces after load that decreases coronary flow [61].

Bioactivation of GTN requires thiols or sulfhydryl containing compounds, and NO or NO-like compounds are believed to be the biologically active species. Repeated administration of GTN produces sulphydryl depletion and the development of tolerance. Mitochondrial aldehyde dehydrogenase (mtALDH) generates 1,2-glyceryl dinitrate and nitrite from GTN. This reaction requires a reducing thiol cofactor. The activity of this enzyme is reduced in GTN tolerance. Once GTN is bioactivated within the mitochondria, nitrite or an additional action of mtALDH generates the vasodilatory NO bioactivity. One suggested mechanism for this vasodilatory activity is that S-nitrosoglutathione is formed by the reaction of reduced glutathione and ni-
trite. This molecule subsequently undergoes biotransformation to S-nitrosocysteine that can release NO [59].

Chronic GTN administration has been shown to result in acetylcholine induced coronary vasoconstriction rather than relaxation [62,63] and induce endothelial dysfunction [64]. GTN is effective in reducing ventricular preload by increasing peripheral venous capacitance. GTN in higher dosages can also decrease pulmonary and systemic vascular resistances. GTN can reduce ventricular filling pressure, wall stress and myocardial oxygen consumption, and may also improve systolic and diastolic ventricular function by improving coronary flow in patients with ischemic cardiomyopathy.

Mitochondrial aldehyde dehydrogenase-2 (ALDH2) plays an important role in the bioactivation of GTN and nitrite. The nitrite formed from GTN metabolism may be further metabolized to NO and/or converted to an S-nitrosothiol. The nitrite anion represents a storage form of NO that can have therapeutic effects.

Both mitochondrial ALDH2 and xanthine oxidoreductase (XOR) can act in a parallel manner to reduce nitrite to vasoactive NO [59] (Figure 7 and Figure 8).

Figure 8. NO formation by endothelial NOS (eNOS), and reduction of nitrite to vasoactive nitric oxide.
3. Aims

3.1. Study I
To study the role of exogenous nitric oxide in inflammatory response in whole blood related to simulated extracorporeal circulation.

3.2. Study II
To study the anti-microbial effects of exogenous nitric oxide on Staphylococcus aureus in whole blood during prolonged simulated extracorporeal circulation.

3.3. Study III
To test the effect of glyceryl-trinitrate on Staphylococcus aureus growth during simulated extracorporeal circulation and also to examine the effect of Staphylococcus aureus, alone and in combination with glyceryl-trinitrate, on activation markers of the innate immune system during simulated extracorporeal circulation.

3.4. Study IV
To study the effect of glyceryl-trinitrate on activation markers of the innate immune system during simulated extracorporeal circulation.
4. Materials and methods

4.1. Study designs
Experimental studies on human WBL during SECC. Study I (n=8 experiments) was randomized, and Study II (n=7 experiments), III (n=5 experiments) and IV (n=5 experiments) were non-randomized. The studies were performed in accordance with the ethical principles in the Helsinki declaration. All studies were conducted within the Department of Surgical Sciences, Section of Cardiothoracic Surgery and Anesthesiology, at Hedenstierna Laboratory (Study I) and at the Blood Central laboratory (Study II-IV), Uppsala University, Uppsala, Sweden. All volunteers giving blood for research were fully informed about the study by investigator (Study I) and according to routines applied at Blood Central laboratory (Study II-IV) (Figure 9).

![Figure 9. Group allocation.](image)

4.2. Preparation of S. aureus
For Study II and III, S. aureus, American Type Culture Collection (ATCC) 29213 was prepared. Before each experiment the strain was grown in 5 mL of Todd-Hewitt broth in plastic tubes at 37°C in air for 5 hours, resulting in approximately 5 x 10^8 colony forming units (cfu)/mL in an exponential growth-phase. The bacterial cultures were diluted 1:10 in phosphate-buffered saline (PBS) just before the start of each experiment.
4.3. Preparation of blood

In all studies, for each experiment, blood was freshly drawn from two clinically healthy human volunteers. In Study I, the blood was stored in heparinized (12.5 IU heparin/mL WBL) collecting bags and then randomly assigned to each of the two groups- NO group and control group. In Study II-IV, the freshly drawn blood was immediately pooled into one large Citrate-Phosphate Dextrose (CPD) bag and thereafter heparinized (1,4-2.8 IU heparin/mL WBL), before entering two separate SECC circuits – NO circuit and control circuit (Study II), and GTN circuit and control circuit (Study III-IV). In Study II-III, prior to entering the SECC circuits, the heparinized CPD blood bag was infested with 1 ml of prepared S. aureus culture to obtain a final concentration of 1x10^4 to 1x10^5 cfu/mL of WBL (Figure 9). The relationship between magnitude of bacteremia and severity of clinical picture has been previously extensively investigated, thus in our model, a final concentration of 1x10^4 to 1x10^5 cfu/mL of WBL would be translated into the clinical range of severe sepsis [65].

4.4. Simulated extracorporeal circulation

In all studies, the SECC circuits were assembled in a standard manner, including a calibrated, barely occlusive Stöckert roller pump (Sorin Group GmbH, München, Germany), PVC tubing and pediatric hollow fiber oxygenator with integral soft-shell venous reservoir D702 Masterflo 51 (Dideco, Mirandola, Italy) (Study I-II) or hard-shell venous reservoir Jostra Quadrox D Bioline (Maquet, Rastatt, Germany) (Study III-IV). The blood was circulated in a closed circuit at 0.5 L/min for 180 minutes (Study I-II) and 240 minutes (Study III-IV). Temperature was maintained at 35°C with a thermostatic heat exchanger (Gambro, Lund, Sweden). The oxygenators were ventilated with an oxygen and air mixture of 40% and 60%, respectively, at 0.4 L/min in Study I and 0.5 L/min in Study II-IV. In Study I-II, the NO SECC circuit was ventilated with an oxygen/air mixture 40%/60% flowing at 0.3 L/min, and NO was delivered through the oxygenator as a mixture of NO and N₂ (AGA Gas AB, Lidingö, Sweden), flow being adjusted to 0.1 L/min to reach, in Study I, an NO concentration of 80 ppm and NO₂ < 4 ppm, and, in Study II, an NO concentration of 85-98 ppm and NO₂ < 2. NO and NO₂ concentrations were continuously monitored with calibrated NoxBox (Bedfont Scientific ltd, United Kingdom) connected to the mixed gas outflow system.

In Study III-IV, GTN (1 mg/mL) was delivered to the SECC GTN circuits at 0.5 mL/h (total amount delivered was 2 mg) by an automated infusion system pump Braun Perfusor (B. Braun, Melsungen, Germany). ACT was measured with Hemotech analyser (Englewood, Colorado, USA), and kept,
in Study I, > 400 s, and, in Study II-IV, > 270 s for the entire duration of the experiments.

Previous studies have shown that heparin may have anti-inflammatory properties by inhibiting leukocyte recruitment [66], rolling and chemotactant-induced firm adhesion [67], and reducing iNOS activity [68]. Also heparin seems to inhibit the enzymatic conversion of nitrate to nitrite [69]. Therefore, in order to reduce these effects, we chose to add less amount of heparin in Study II-IV. As to thrombin formation, an ACT rate of between 180 to 220 seconds is considered to result in adequate anticoagulation during ECC [70]. Blood gases and pH were measured in the AVL Omni Modulator System (AVL Scientific Corp, Roswell, GA, USA) with mean levels of PO2 35.3 kPa, PCO2 1.6 kPa, and pH 7.5.

4.5. Experimental procedure and measurements

4.5.1. Blood samples

In Study I, blood samples were taken at four different time-points; pretreatment, 60, 120 and 180 minutes of SECC. The first sample was taken directly from the donor’s vein and the subsequent blood samples were taken from the circuit to the tubes.

In Study II, blood samples were taken at six different time-points; immediately after heparinization and infestation with S. aureus, then at 3, 30, 60, 120 and 180 minutes of SECC.

In Study III, blood samples were taken at five different time points; immediately after heparinization and infestation with S. aureus, then at 30, 60, 180, and 240 minutes of SECC.

In Study IV, blood samples were taken at five different time points; promptly after heparinization and then at 30, 60, 180, and 240 minutes of SECC.

In Study II-IV, the first blood sample was taken directly from the CPD bag and the subsequent samples from the SECC circuits.

In all studies, samples for blood cells count and leukocyte differential were collected in tubes containing 7.2 mg ethylene-diamine-tetra-acetic acid (EDTA). Samples for MPO and HNL were drawn into serum separating (SST) tubes, centrifuged in a cooled centrifuge (Study I: 1300 rpm, Study II-IV: 2000 rpm) for 10 min and the plasma immediately frozen (Study I: –20°C, Study II-IV: –70°C) until measurements were done. In Study II-IV, samples for C3a and TCC were collected in EDTA tubes, then centrifuged (2000 rpm, 10 min) and the plasma immediately frozen and stored at –70°C for later quantitative analysis.
4.5.2. Measurement of granulocyte oxygen free radical production

In Study I, blood samples for oxygen free radical measurements in WBL and isolated granulocytes were drawn into tubes with 15 IU sodium heparin/mL. Granulocyte isolation was started with mixing 5 mL heparinized blood with equal volume of 2% (w/v) dextran solution. Red blood cells sedimented in polypropylene tubes for 30 minutes at room temperature. Then, leukocyte supernatant was centrifuged (1500 rpm, 5 min) and cells were resuspended in 0.9 % NaCl. Thereafter 6 mL sterile water was added for 30 seconds and after addition of 2 ml 3.6 % NaCl cells were spun down (1500 rpm, 5 min), supernatant and erythrocyte membranes removed, and poly – and mononuclear leukocytes counted. Just before the analysis, Gey’s solution was added to achieve a granulocyte concentration of 1x10⁹ cells/L. Capacity of WBL as well as capacity of isolated granulocytes to release ROS were estimated by chemiluminescence (CL) in WBL and in isolated polymorphonuclear leukocytes (PMN) after stimulating with either serum opsonized zymosan (SOZ) or phorbol myristate acetate (PMA). Two different chemiluminescent probes, lucigenin and luminol, were applied. CL assay in WBL was performed after adding 50 µL blood and 400 µL Gey’s solution and incubated at 37³C. Luminol (0.1 mL, 1 g/L) (Kebo Biomed, Stockholm, Sweden) or lucigenin (0.1 mL, 0.5 g/L) (Sigma Chemical, St Louis, USA) was added. After incubating the mixture during five more minutes 0.1 mL of stimulating agent SOZ (4 g/L, room temperature) (ICN Biochemicals, Cleveland, USA) or PMA (16 nM/L, 4° C) (Sigma Chemical, St. Louis, USA) was administered. CL activity was recorded continuously for the period of 10 minutes with Biocounter RM 2010 (Lumac B.V., Netherlands). In isolated granulocyte CL assay instead of blood 0.1 mL cells with concentration 1x 10⁹ cells/L was mixed with 0.1 mL SOZ or PMA. After addition of 0.1 mL luminol (0.025 g/L) or lucigenin (0.1 g/L) CL was recorded. Curve peak was registered for luminol and the value after 10 minutes for lucigenin. The values were expressed as counts per minute (CPM). Areas under curve (AUC) were determined. CL values for WBL were corrected for the Hb concentration and number of granulocytes. CPM were calculated at Hb concentration of 100 g/L and in relation to the concentration of granulocytes; CPM/ 10⁶ granulocytes.

4.5.3. Analyses of cytokines, HNL, LF, MPO, C3a, TCC and nitrite/nitrate

Samples for cytokine and HNL, LF, and MPO measurements were collected into EDTA tubes, and nitrite/nitrate samples were taken into tubes without any anticoagulants. After centrifugation plasma/serum samples were frozen immediately to −70°C.
In all studies, MPO in plasma was detected with radioimmunoassay kit according to manufacturer’s recommendation (Pharmacia Diagnostics AB, Uppsala, Sweden), and quantified in Wallac 1260 Multigamma Counter (Wallac Oy, Turku, Finland).

In Study I, HNL in plasma was determined with a double radioimmunoassay and LF level was measured using standard ELISA kit (R&D Systems, Minneapolis, USA).

Quantitative analysis of plasma C3a and TCC was performed with a Biacore 2000 instrument (BiaCore AB, Uppsala, Sweden). Blood samples for cytokines and nitrite/nitrate were collected from the circuit and centrifuged. Then plasma samples were frozen at –70°C until assays were performed. Concentrations of IL-1β and IL-10, were measured by specific enzyme-linked immunoabsorbent assay (Boehringer, Mannheim, Germany). Serum levels of nitrite/nitrate were measured according to the procedure based on the Griess reaction after the enzymatic reduction of nitrate to nitrite (Nitric Oxide Calorimetric Assay Kit, R&D Systems, Minneapolis, USA). First serum was ultrafiltrated by centrifugation (6000 rpm, 60 min, 10°C) using 10 000 MW filters (Ultratfree-MC, Millipore, France). Nitrate was reduced to nitrite by adding nitrate reductase NADPH and Flavin-adenine-dinucleotid (FAD). The nitrite was measured by addition of Griess reagents (sulphanilamide and N-(1-naphtyl)-ethylene diamine dihydrochloride) and the absorbance was read at a wavelength of 540 nm using microtitre plate reader. Nitrite concentration was calculated by comparison with the standard curve. The results reflected total nitrite plus nitrate concentrations and were expressed in micromole of NO₂⁻/NO₃⁻ per litre of plasma. All measurements were performed in duplicate.

4.5.4. Measurement of blood cell counts
Hb, leukocyte and leukocyte subset counts were determined with an automatic cell counter (Coulter Counter, Florida, USA).
In Study II-IV, Hb, platelet, leukocyte and leukocyte subset counts were determined with an automatic cell-counter (Medonica CA 620 Loke 16-parameter system).

4.5.5. Preparation of leukocytes for flow cytometry analyses
In Study III-IV, leukocytes were prepared as previously described (71). Briefly, 1mL heparinized blood was fixated with 0.4% paraformaldehde and the erythrocytes were lysed by incubation with 0.85% (w/v) NH4Cl in Tris-HCl buffer [Tris(hydroxymethyl)-aminomethane 0.01 mol/L, pH 7.4]. Finally, the cells were washed with PBS containing human serum albumin (HSA) (0.1%, w/v) and diluted to the concentration of 2.5x10⁶/mL.
4.5.6. Labeling of leukocytes with antibodies to cell surface antigens for flow cytometry analyses

In Study III-IV, 50 μL samples of the leukocyte suspension were mixed with optimally titrated fluorescein isothiocyanate (FITC)-, phycoerythrine-, or unlabeled mouse monoclonal antibodies (mab) against CD11b, CD35, CD63, CD64, CD65, CD66b (Beckman Coulter, Fullerton, California, United States), CD16, CD14, (Dako, Glostrup, Denmark), and CD32 (Becton-Dickinson, San Diego, California, United States) and incubated for 30 minutes at 4°C. After incubation, the cells were washed with PBS and thereafter diluted in 300 μL PBS with HSA. Leukocytes were also labeled by an identical procedure with negative isotype controls for mouse immunoglobulin G1 (IgG1) and IgG2 (Dako). After labeling, the cells were kept on ice until analysis. The high non-specific background that may be caused by the multiple FC-receptors of the granulocytes is reduced by this procedure [71,72].

4.5.7. Flow cytometry

In Study III-IV, flow cytometric analysis was performed on an EPIC MCL-XL flow cytometer (Beckman Coulter). Identification of granulocytes was based on their forward scatter/side scatter (FSC/SSC) dot-plot profile and of monocytes on the FSC/SSC dot-plot profile and positive staining with anti-CD14. The granulocyte and monocyte populations were gated and the FITC fluorescence measured. The fluorescence intensity above background (isotype control) of granulocytes and monocytes was determined and expressed as mean fluorescence intensity. In case of CD35 and CD64, expression was also given as relative number of positive cells (%), defined as the relative number of cells that expressed CD35, respectively, CD64 to a higher extent than background (isotype control).

4.5.8. Bacteriological analysis

In Study II, blood samples (5mL) from the control and NO circuits were collected at 0, 3, 30, 60, 120 and 180 minutes. In Study III, blood samples (5 mL) from the infected GTN and control circuits were collected at 0, 30, 60, 180, and 240 minutes. The samples were serially diluted in PBS. At least three samples (10 or 100 µl) from the original bacterial suspension were subsequently spread on agar plates and incubated for two days at 37°C in the adequate environment, spread on agar plates, and incubated for two days at 37°C. The quantification of bacterial growth was determined by manual count of the number of colony forming unit, according to clinically validated routines.
5. Statistics

5.1. Study I
A two-way repeated measures analysis of variance (ANOVA) was carried out for each of the outcomes. In each case there was one repeated factor (Time) and one non-repeated factor (Group). The results of the analysis determined if there was strong evidence in the data that: 1. the mean outcome value differed between two groups, 2. the mean outcome value differed over the four time points, and 3. the average difference between the two groups changed from one time point to another (i.e. interaction effect). Bonferroni correction was applied because multiple tests were conducted, thereby insuring that all results that are declared to be statistically significant were in fact significant at an overall significance level of 0.05.

5.2. Study II
Data are presented as mean +/- SEM from 7 independent paired experiments. Statistical analysis was performed using student t-test, and p values < 0.05 were considered significant.

5.3. Study III
Data are presented as median and range from five independent experiments. Statistical analysis was performed using nonparametric ANOVA (Kruskal–Wallis), and p values < 0.05 were considered significant. All the statistical analyses were performed with the Statistica 10.0 software for Windows (StatSoft, Tulsa, Oklahoma, United States).

5.4. Study IV
Data are presented as median and range from five independent experiments. Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis), and p values 0.012 or less were considered significant (corrected for multiple comparisons). All the statistical analyses were performed with the Statistica 10.0 software for Windows (StatSoft, Tulsa, Oklahoma, United States).
6. Results

6.1. Findings in Study I

There were four outcomes for which there was a statistically significant interaction effect: MPO, HNL, LF and nitrite/nitrate. There was no significant difference between the groups at baseline but thereafter the difference between the groups steadily increased with time. Also, for every outcome except for three there was a significant time effect; i.e. the mean outcome was not the same for all time points. A time effect was not observed for IL-1β release and peak values for superoxide production in whole blood and isolated granulocytes after stimulation with SOZ and using lucigenin probe.

6.1.1. Granule release

MPO, HNL and LF release were increased after nitric oxide addition into the circuit. There was a time effect and an interaction effect for all measured granule derived proteins; MPO, HNL and LF. For MPO release the average difference in outcome between NO group and control group increased from one time point to the next, interaction effect (p=0.0001). In every case, where the difference was statistically significant, the mean outcome was higher in NO group than in control group. The difference was not significant at 60 minutes but became highly significant at 120 minutes (p=0.017) and 180 minutes (p=0.008) (Figure 10A). MPO concentration started in group NO from 290±170 μg/L and reached 2191±903 μg/L (p<0.0002). In group C the initial MPO level was 350±223 μg/L and increased to 1036±366 μg/L (p<0.0002). The average difference in outcome for HNL release between the NO group and controls increased from one time point to the other i.e. interaction effect (p=0.004). In every case, when the difference was statistically significant, the HNL release was higher in the NO group than in the control group. The difference was not statistically significant at 60 minutes; it was marginally significant at 120 minutes (p=0.075) and at 180 minutes (p=0.052) (Figure 10B). HNL release increased in group NO from 88±39 μg/L to 431±169 μg/L (p<0.0002) and in group C from 86±20 μg/L to 287±63 μg/L (p<0.0002). LF levels increased in both groups – in group C from 117±134 μg/L to 1600±886 μg/L (p<0.0002) and in group NO from 92±43 μg/L to 2574±1048 μg/L (p<0.0002). The mean difference between the two groups became marginally significant at 180 minutes (p=0.07) (Fig-
The mean difference between groups is not significant at the other time points (p>0.3). The mean difference in outcome between groups increased from one time point to the next.

6.1.2. Nitrite/nitrate

Mean response in control group remained constant over time. Addition of NO caused an increase in nitrite/nitrate concentration and an interaction effect was recorded (p=0.0001). There was a significant difference between the groups (p=0.0001) except for baseline values. In fact, the difference in mean response between NO group and control group increased steadily over time (Figure 10D).

*Figure 10. Plasma concentrations of A) MPO, B) HNL, C) LF, and serum concentrations of D) nitrite/nitrate. Values are mean ± SD. “·”= group NO (with nitric oxide addition), “Δ” = group C (control group), ’= minutes, MPO = myeloperoxidase, HNL= human neutrophil lipocalin, LF= lactoferrin, SECC = simulated extracorporeal circulation, * = p=0.017, ** = p=0.008, *** = p=0.0001, comparison between the groups.*
6.1.3. Oxygen free radical producing capacity

Capacity to release ROS was measured as peak values, corrected peak values and AUC for WBL and isolated granulocytes after stimulation with SOZ or PMA using lucigenin and luminol as probes. Results are reported in AUC, which is biologically the most representative parameter (Table 1). Circulating ROS levels were not measured. WBL lucigenin CL assay determining $O_2^-$ release after SOZ stimulation revealed a decrease over time (p<0.0002). In isolated PMN lucigenin assay using SOZ stimulation after an initial increase a decrease was recorded towards the end of the experiment (p=0.0003). Similarly, isolated PMN $O_2^-$ release measured after PMA stimulation showed a decrease over time (p=0.01). There was no statistically significant difference (p>0.05) between the groups. WBL ROS release measured with luminol assay after SOZ stimulation increased initially, thereafter a decrease occurred (p<0.0002). There was no statistically significant difference between the groups. Isolated PMN ROS release recorded with luminol assay after stimulation with SOZ (p=0.006) and PMA (p<0.0002) revealed a decrease in ROS production capacity towards the end of the experiment. No statistically significant difference (p>0.05) was found between the groups.
Table 1. Changes in oxygen free radical producing capacity. Results (mean ± SD) reported as AUC. * = minutes. AUC = area under curve. luc = lucigenin. lum = luminol. NS = no significance. p1 = comparison within the group (180' sample vs 0' sample). p2 = comparison between the groups. PMA = phorbol myristate acetate. PMN = polymorphonuclear leukocytes. SECC = simulated extracorporeal circulation. SOZ = serum opsonized zymosan. WBL = whole blood.

<table>
<thead>
<tr>
<th>SECC</th>
<th>0'</th>
<th>60'</th>
<th>120'</th>
<th>180'</th>
<th>p-value¹</th>
<th>p-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBL SOZ-luc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group N</td>
<td>7.6 ± 4.3</td>
<td>6 ± 2.8</td>
<td>5 ± 2.3</td>
<td>4.4 ± 1.6</td>
<td>&lt; 0.05</td>
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</tr>
<tr>
<td>Group C</td>
<td>6.8 ± 3.4</td>
<td>6.3 ± 2.5</td>
<td>5.4 ± 1.8</td>
<td>5.5 ± 1.6</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>PMN SOZ-luc</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Group N</td>
<td>15.2 ± 4.1</td>
<td>15.5 ± 3.4</td>
<td>13.5 ± 4.3</td>
<td>9.9 ± 5.2</td>
<td>&lt; 0.05</td>
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</tr>
<tr>
<td>Group C</td>
<td>14.4 ± 3.5</td>
<td>16 ± 3.2</td>
<td>15.5 ± 5.4</td>
<td>9.5 ± 3.8</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>PMN PMA-luc</td>
<td></td>
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<td></td>
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<tr>
<td>Group N</td>
<td>21.6 ± 6.8</td>
<td>26 ± 12</td>
<td>23.3 ± 17</td>
<td>11.4 ± 4.3</td>
<td>&lt; 0.05</td>
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<tr>
<td>Group C</td>
<td>26.1 ± 15.7</td>
<td>37.1 ± 23.3</td>
<td>35.7 ± 23.7</td>
<td>20.1 ± 8.8</td>
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<td>NS</td>
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<tr>
<td>WBL SOZ-lum</td>
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<tr>
<td>Group N</td>
<td>4.9 ± 1.6</td>
<td>9.5 ± 3.1</td>
<td>7.8 ± 3.4</td>
<td>6.5 ± 2.1</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td>6.5 ± 2.6</td>
<td>10.9 ± 4.2</td>
<td>9.2 ± 2.1</td>
<td>8.8 ± 2.5</td>
<td></td>
<td>NS</td>
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<tr>
<td>PMN SOZ-lum</td>
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<tr>
<td>Group N</td>
<td>8.3 ± 1.8</td>
<td>8.6 ± 1.6</td>
<td>7.7 ± 1.9</td>
<td>6.7 ± 2.1</td>
<td>&lt; 0.05</td>
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<tr>
<td>Group C</td>
<td>9.4 ± 2.6</td>
<td>9.7 ± 1.7</td>
<td>9.8 ± 2.3</td>
<td>6.4 ± 2.6</td>
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<td>NS</td>
</tr>
<tr>
<td>PMN PMA-lum</td>
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<tr>
<td>Group N</td>
<td>2.7 ± 0.8</td>
<td>2.5 ± 0.9</td>
<td>1.9 ± 0.8</td>
<td>1.3 ± 0.6</td>
<td>&lt; 0.05</td>
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</tr>
<tr>
<td>Group C</td>
<td>3.3 ± 2.4</td>
<td>3.1 ± 1.3</td>
<td>2.4 ± 0.9</td>
<td>1.6 ± 1.0</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

6.1.4. White blood cell (WBC) and platelet counts and differential

WBC, neutrophil count and monocyte count decreased over time (p<0.0002) but there was no statistically significant difference (p>0.05) between the groups. Platelet count decreased after initiation of the circuit and there was no statistically significant difference between the groups (p>0.05). Lymphocyte count and Hb increased over time (p<0.0002) (Table 2).
Table 2. Changes in hemoglobin and leukocyte subsets. Values are means ± SD. ‘ = minutes. Hb = hemoglobin. NS = no significance. p1 = comparison within the group (180’ sample vs 0’ sample). p2 = comparison between the groups. SECC = simulated extracorporeal circulation. WBC = white blood cells count.

<table>
<thead>
<tr>
<th></th>
<th>0’</th>
<th>60’</th>
<th>120’</th>
<th>180’</th>
<th>p1</th>
<th>p2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WBC (10^9/L)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Group N</td>
<td>4.28 ± 1.38</td>
<td>4.16 ± 1.3</td>
<td>3.74 ± 1.2</td>
<td>3.76 ± 1.27</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Group C</td>
<td>4.48 ± 1.4</td>
<td>4.41 ± 1.32</td>
<td>3.96 ± 1.21</td>
<td>3.93 ± 1.27</td>
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</tr>
<tr>
<td><strong>Neutrophils (10^9/L)</strong></td>
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<td></td>
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</tr>
<tr>
<td>Group N</td>
<td>2.39 ± 0.76</td>
<td>2.12 ± 0.6</td>
<td>1.74 ± 0.61</td>
<td>1.65 ± 0.54</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Group C</td>
<td>2.79 ± 0.69</td>
<td>2.46 ± 0.56</td>
<td>2.15 ± 0.59</td>
<td>2.14 ± 0.53</td>
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<tr>
<td><strong>Monocytes (10^9/L)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Group N</td>
<td>0.35 ± 0.06</td>
<td>0.29 ± 0.08</td>
<td>0.21 ± 0.06</td>
<td>0.19 ± 0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Group C</td>
<td>0.39 ± 0.12</td>
<td>0.34 ± 0.12</td>
<td>0.24 ± 0.08</td>
<td>0.22 ± 0.07</td>
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<tr>
<td><strong>Platelets (10^9/L)</strong></td>
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<tr>
<td>Group N</td>
<td>240 ± 48</td>
<td>105 ± 29</td>
<td>113 ± 29</td>
<td>121 ± 30</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Group C</td>
<td>235 ± 26</td>
<td>60 ± 22</td>
<td>101 ± 26</td>
<td>111 ± 25</td>
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<tr>
<td><strong>Lymphocytes (10^9/L)</strong></td>
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<td></td>
</tr>
<tr>
<td>Group N</td>
<td>1.33 ± 0.54</td>
<td>1.58 ± 0.67</td>
<td>1.5 ± 0.7</td>
<td>1.72 ± 0.7</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Group C</td>
<td>1.42 ± 0.25</td>
<td>1.50 ± 0.32</td>
<td>1.63 ± 0.29</td>
<td>1.78 ± 0.39</td>
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<tr>
<td><strong>Hb (g/L)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group N</td>
<td>130 ± 13</td>
<td>143 ± 14</td>
<td>144 ± 15</td>
<td>144 ± 15</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Group C</td>
<td>142 ± 13</td>
<td>146 ± 13</td>
<td>146 ± 15</td>
<td>145 ± 15</td>
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</tr>
</tbody>
</table>

6.1.5. Interleukin 1-beta and Interleukin 10

There was no significant change in IL-1β levels over time (p=0.4906). However, IL-10 levels increased during the experiment after initial decrease (p=0.007). Peak was reached at the end of the experiment. NO administration did not affect either IL-1β or IL-10 levels.

6.2. Findings in Study II

In the control SECC circuit, the absolute number of S. aureus cfu dropped to 39% after 60 min and returned back to 76% of starting amounts after 180 min. This indicates initial bacterial death during the first hour on SECC and subsequent partial recovery at 180 min. In the NO circuit, no significant changes in the number of S. aureus cfu was observed up until 120 min, but it
increased significantly by 180 min, compared to starting amount. Furthermore, the number of S. aureus cfu were significantly higher in the NO circuit after 60 min, which subsequently increased by 6.2-fold (p=0.002) compared to control, after 180 min on SECC (Figure 11). This suggests that NO improves S. aureus survival during the early time-points and enhances growth at the later time-point.

![Figure 11. Growth of S. aureus in SECC, control (●) and with NO (○). The data is presented as the mean of the number of S. aureus cfu +/- SEM (n=7). * = p<0.05, ** = p<0.01, comparison between the groups.](image)

The leukocyte counts remained constant during the entire SECC run in both NO and control circuits.

MPO release, C3a and TCC levels were measured to assess the leukocyte response to SECC and S. aureus. A steady increase in both MPO, C3a and TCC level was observed during the entire length of each experiment. At 180 min, there were significant increases by 8-fold in MPO level and by 2-fold in both C3a and TCC levels compared to starting levels. However, there were no differences in MPO, C3a and TCC levels between control and NO circuit (Figure 12, A-C).
6.3. Findings in Study III

S. aureus were introduced into parallel SECC circuits. GTN was added to one of these parallel circuits. We performed five independent parallel SECC runs, each lasted 240 minutes. In both groups, bacterial counts were significantly reduced (p<0.05) over time. To explore possible leukocyte mediated immunological mechanisms involved in the potential reductive effect of GTN on S. aureus growth, we analyzed a panel of markers of inflammatory response.

S. aureus significantly reduced the expression of granulocyte CD32 and CD162, and stimulated the expression of granulocyte and monocyte CD11b, monocyte CD14, monocyte CD32, leukocyte CD55, monocyte CD63, monocyte CD65, granulocyte CD66b and monocyte CD162, and increased levels of TCC, C3a and MPO (p<0.05) over time.

GTN did not affect S. aureus growth during SECC (Figure 13) and had no effect on SECC-induced leukocyte activation (Figure 14-17).
6.3.1. Staphylococcus aureus growth

![Graph showing S. aureus growth](image)

*Figure 13.* Growth of S. aureus during SECC, without (○) and with GTN (●). Data are presented as the median of the number of S. aureus CFU/mL whole blood with range (n=5).

6.3.2. Leukocyte Fcγ receptors

S. aureus significantly reduced the expression of granulocyte CD32 and stimulated the expression of monocyte CD32 (p<0.05) over time (*Figure 14, A and B*). Theoretically, the reduced expression of granulocyte CD32 may be due to an internalization of the receptor. There were no significant changes, with or without addition of GTN, for the expression of leukocyte CD16 and CD64. Addition of GTN to the circuits did not generate any significant intergroup changes in the expression of CD32.
Figure 14. Changes in levels of leukocyte Fcγ receptors during SECC, S. aureus infested without (○) and with GTN (●). Expression of A) granulocyte CD32 and B) monocyte CD32. Data are presented as median fluorescence intensity (MFI) value with range (n=5).
6.3.3. Main leukocyte adhesion CD molecules

Expression of key molecules involved in cell adhesion was determined by measuring the level of CD11b (adhesion and C3bi receptor), CD65 (E-selectin ligand), and CD162 (P-selectin ligand). Addition of *S. aureus* induced significant increases in expression of granulocyte and monocyte CD11b, monocyte CD65, and monocyte CD162 over time (p<0.01), indicating an activation of these cells (*Figure 15, A-D*). Granulocyte expression of CD162 was though reduced over time. Addition of GTN to the circuits did not generate any significant intergroup changes in the expression of CD11b, CD65, and CD162.
6.3.4. LPS-receptor, C3b-receptors and leukocyte activation marker

To screen for other potential effects of GTN on other components of the innate immune system, we also analyzed the levels of CD14 (lipopolysaccharide receptor), CD35 (C3b receptor), and CD66b (a marker of granulocyte activation). Addition of S. aureus induced significant increases in monocyte CD14, leukocyte CD35, and granulocyte CD66b levels (p<0.01) over time. Addition of GTN to the circuits did not generate any significant intergroup changes in the expression of CD14, CD35, and CD66b (Figure 16, A-D).
A

B
Figure 16. Changes in levels of components of the innate immune system during SECC, S. aureus infested without (○) and with GTN (●). Expression of A) monocyte CD14, B) granulocyte CD35, C) monocyte CD35, and D) granulocyte CD66b. Data are presented as median fluorescence intensity (MFI) value with range (n=5).
The levels of granulocyte CD63 (lysosomal granule protein) were analyzed. Addition of S. aureus resulted in a significant increase in monocyte CD63 levels (p<0.01) over time. Addition of GTN to the circuits did not generate any significant intergroup changes in the expression of CD63 (Figure 17).

![Monocyte CD63](image)

*Figure 17. Changes in leukocyte expression of lysosomal granule membrane protein CD63 during SECC, S. aureus infested without (○) and with GTN (●). Data are presented as median fluorescence intensity (MFI) value with range (n=5).*

6.3.5. MPO, TCC and C3a

S. aureus induced a significant increase of TCC, C3a, and MPO levels (p<0.01) over time. Addition of GTN to the circuits did not generate any significant intergroup changes for TCC, C3a, and MPO.

6.4. Findings in Study IV

SECC stimulated the expression of monocyte LPS-receptor CD14 and C3b-receptor CD35. GTN significantly reduced the expression of leukocyte Fcγ receptor CD32 over time, compared to control. SECC increased concentrations of MPO, TCC, complement component C3a. Addition of GTN did not significantly affect these changes.
6.4.1. Leukocyte Fcγ receptors
Changes in three Fcγ receptors were evaluated by measuring CD16, CD32 and CD64. The expression of granulocyte CD32 was not significantly affected by SECC but the presence of GTN significantly reduced granulocyte expression of CD32 (p<0.01) over time, compared to control (Figure 18).

Figure 18. Changes in levels of granulocyte Fcγ receptors during SECC, without (○) and with GTN (●). Expression of granulocyte CD32. Data are presented as median fluorescence intensity (MFI) value with range (n=5).

6.4.2. Main leukocyte adhesion CD molecules
By measuring the level of CD11b (adhesion and C3bi receptor), CD65 (E-selectin ligand) and CD162 (P-selectin ligand), we determined the expression of key proteins involved in cell-adhesion. There were no significant changes in the expression of these markers.

6.4.3. LPS-receptor, C3b-receptors and leukocyte activation marker
The levels of CD14 (LPS-receptor), CD35 (C3b-receptor) and CD66b (a marker of leukocyte activation) were analysed to screen for potential effects of GTN on other components of the innate immune system. SECC induced significant increases in monocyte CD14 (p<0.01) and CD35 levels (p<0.001) over time. Addition of GTN did not generate any significant inter-group differences (Figure 19, A and B).
Figure 19. Changes in levels of components of the innate immune system during SECC, without (○) and with GTN (●). Expression of A) monocyte CD14 and B) monocyte CD35. Data are presented as median fluorescence intensity (MFI) value with range (n=5).
6.4.4. MPO, TCC and C3a
SECC induced significant increase in levels of MPO, TCC and C3a (p<0.001) over time. Addition of GTN to the circuits did not generate any significant inter-group differences (Figure 20 and Figure 21, A to B).

![Graph showing changes in MPO levels during SECC with and without GTN.](image)

*Figure 20. Changes in myeloperoxidase (MPO) levels during SECC, without (○) and with GTN (●). Data are presented as median concentration value with range (n=5).*
Figure 21. Changes in parameters of the innate immune system during SECC, without (○) and with GTN (●). A) Terminal complement complex (TCC). B) Complement component 3a (C3a). Data are presented as median concentration value with range (n=5).
6.4.5. Blood leukocyte counts
Measurement of leukocyte concentrations during SECC showed significantly reduced levels (p<0.01) over time. Addition of GTN to the circuits did not generate any significant inter-group differences.
7. Discussion

In the cardiopulmonary bypass circuit, blood is exposed to artificial surfaces, and together with other factors such as anesthesia, drugs, surgical trauma, hypothermia-rewarming, endotoxemia and IR, cause activation of both cellular and non-cellular blood components and induce SIR. This reaction has vast similarities to inflammatory reaction in sepsis caused by bacteria, fungi, and viruses and they result in tissue injury through a variety of mediators and direct activation of leukocytes and endothelial cells. The rate-limiting step in inflammation is leukocyte adhesion to endothelium and it is regulated by various factors, such as expression of adhesion molecules on leukocytes and endothelial cells, free radicals derived from endothelium and leukocytes, and shear forces. Oxidants and proteases derived from PMN cause the cellular injury and dysfunction in tissues by damaging DNA and membrane lipids [73].

NO is described as having both pro-inflammatory and anti-inflammatory qualities and to exert both beneficial and deleterious effects in different tissues under different conditions. Endothelial cell derived NO modulates leukocyte-endothelial cell interaction by influencing integrins and/or formation of focal adhesions [73-75]. Proposed anti-inflammatory mechanisms of NO includes modulating adhesion molecule expression on PMN and endothelial cells, decreasing vascular permeability, scavanging superoxid anion in endothelial cells, and inhibiting PMN NADPH oxidase thus causing reduction in superoxide anion formation. It has been reported that NO production is increased during cardiopulmonary bypass and iNOS is known to be induced by cytokines during SIR [76,77].

On the other hand, it has been proposed that NO is detrimental and involved in tissue injury. Acute lung injury and inhibition of pulmonary surfactant function and myocardial injury may be caused by peroxynitrite and peroxynitrate intermediates formed in a reaction between superoxide and NO [73, 78]. Concerning reperfusion the evidence is inconclusive and there are reports that NO may be protective or that it may be deleterious. Myocardial contractile dysfunction in sepsis seems to be cytokine mediated through either constitutive NOS (cNOS) or iNOS activation [79,80].

Our ex vivo SECC model is devoid of interaction between blood and endothelial cells, does not permit turnover of cells and exposes blood for considerable trauma, thus enabling us to study the events occurring in the setting of specific milieu, where there are no new cells entering the circulation, no
interaction occurs with other body cells, and the exposure to the biomaterials as well as to investigated factors (NO, GTN, S. aureus) is to much higher degree under investigators control.

In Study I, the effects of NO on leukocyte response were evaluated during SECC by determining release of leukocyte granules and interleukins in the circuit and evaluating ROS producing capacity in vitro. NO is the probable cause of increased release of leukocyte derived proteins MPO, LF and HNL, and its presence in the circuit was confirmed by the increase of NO metabolites nitrite/nitrate. HNL and LF are specifically released from granulocytes and MPO can derive from both granulocytes and monocytes. The effect on MPO release was significant earlier than HNL and LF, so the granule proteins were concluded to derive from both monocytes and granulocytes. However, it could be also explained by earlier release of primary than secondary granules. Biologic explanation for increased granule protein concentrations could be either cell lysis or increased granule secretion during leukocyte activation and adhesion to the foreign surfaces. Hb and lymphocyte counts increased and there was no statistically significant difference between the groups in white blood cell, neutrophil, monocyte counts and fractions, which prevent one from concluding that NO induced toxic lysis is a cause for increased granule release.

The results of Hb and lymphocyte counts may be due to measurement’s margins of fault or due to sampling protocol; in Study I 1st measurements were done in blood taken directly from donors vein. Following samples were from circuits (absolute values at these times were not highly different). The increase in Hb is probably not even significant, considering the small difference between the mean values and the relatively high SD. The increased lymphocyte counts could also be an artefact due to aggregates of platelets and/or apoptotic leukocytes.

NO is suggested to be both a scavenger of oxygen free radicals and an inhibitor of NADPH-oxygenase thus theoretically decreasing measured ROS release values. In our study, we did not find supporting evidence for the inhibition of NADPH oxidase, and while increasing granule secretion, NO virtually did not affect ROS or interleukin production. However, it is important to notice that instead of measuring oxygen free radical levels in circulating blood we determined the PMN and WBL capacity to release ROS after stimulation with PMA or SOZ in vitro. In WBL, the recorded AUC in lucigenin assay after SOZ stimulation revealed decreasing superoxide production capacity and in luminol assay after elevation at 60 minutes a decreasing capacity to produce other ROS was noticed. This phenomenon may have two explanations - it could be interpreted as exhaustion of WBL capacity to produce ROS during longer duration of SECC, or as tolerance induction to foreign surfaces by anti-inflammatory chemokines such as IL-10. Also in isolated PMN, decreasing capacity to produce ROS after an initial increase was noticed. Addition of NO had not demonstrated any effect on the
oxygen free radical production. There are some possible explanations for the lack of NO effect on ROS release. First, it could be explained that the half-life of NO may have been too short to affect ROS production when measurements were made outside of circuit. Second, the initiation of ROS release may require a higher NO dose than for granule release. Third, NO could have reacted to form some other compound with oxygen free radicals, which was not measured and so neutralized the measured oxygen free radicals. The resulting compound could be peroxynitrate, formed in the reaction between NO and oxygen free radicals. The fourth explanation could be increased IL-10 levels, inhibiting the production of ROS.

Interleukin release during SECC is controversial both regarding the time point of elevation and applicability of results to clinical bypass [81]. In general, it is proposed that the cytokines peak several hours after CPB and the main effect is thought to be in the postoperative period [73]. IL-1β is a pro-inflammatory endogenous pyrogen produced by monocytes, endothelial cells and some epithelial cells. In this study no significant difference in the interleukine-1β levels over time or between the groups could be shown. This result could be explained by the in vitro SECC model itself; there is probably no IL-1β production due to the lack of endothelial-and epithelial cells and macrophages. For monocytes to produce IL-1β in an in vitro model, the cultivation time required would be much longer (minimum 24 hours) than in Study I. IL-10 is an anti-inflammatory cytokine and is produced by T-cells, B-cells and monocytes/macrophages. IL-10 has various effects such as suppression of T-cell cytokine synthesis both on protein and mRNA level, and down regulation of monocyte HLA-DR expression and NO-production. Generally, it is an inhibitor of T\textsubscript{h}1 response [82]. IL-10 levels increased after an initial decrease and peaked at the end of the experiment. The source of this is probably IL-10 stored in monocytes and/or lymphocytes. Newly produced IL-10 is not detectable before seven hours [83]. NO had no effect on measured interleukin production. Pro-inflammatory IL-1β could not cause the increased leukocyte granule release because IL-1β levels did not change during the experiment and no difference between the groups was recorded. Increased IL-10 levels could theoretically decrease leukocyte reactivity and increase tolerance towards the circuit surfaces. IL-10 elevation occurred simultaneously with the decreasing oxygen free radical producing capacity and IL-10 is known to inhibit production of ROS and reactive nitrogen intermediates by macrophages [82,84]. This is an alternative explanation to PMN exhaustion in oxygen free radical production. If increased granule derived protein levels were caused by release from activated adhered leukocytes and not lysis it means that IL-10 would selectively reduce oxygen free radical production capacity without at the same time affecting leukocyte granule release. However, other cytokines not measured in this study could also be involved in this process.
Decrease in the WBC count, neutrophil count and monocytes could be interpreted either as a respective cell type adhering to the artificial surfaces of SECC circuit or increased lysis of the respective cell type. Decrease in platelet count was interpreted as platelet adhesion to the artificial surfaces in the circuit. Hb and lymphocyte count did not fall thus excluding general toxic nitric oxide induced lysis as a cause for changes in PMN and monocyte counts and function. NO had no effect on cell counts or fractions.

Some studies show that generation of NO is an important broad-spectrum mechanism in host defense against microorganism [85-90]. In human, S. aureus stimulates NO production by phagocytes and increased NO production is observed in patients with the infection [91,92].

Deep sternal SSIs are a severe complications after cardiac surgery, with associated mortality ranging from 15% to 40% [93-95]. In more than one third of these infections, the isolated pathogens are S. aureus [96]. Previous studies have identified a number of patient-related risk factors, such as diabetes mellitus and obesity [97,98], but also ‘patient independent’ factors, such as surgical techniques, hygiene measures and causative pathogens, may have a significant impact on the overall risk of postoperative SSIs [96].

Having seen some pro-inflammatory effects of NO in SECC, and after taking in account the reports of NO’s antibacterial potential, we therefore hypothesized, in Study II, that addition of NO to SECC may suppress S. aureus growth. We have shown, in Study II, that S. aureus growth is suppressed in the SECC environment. This is presumably because of the overall increased oxidative stress and activation of leukocytes in this mechanical system [99-101]. The addition of NO to SECC circuit did not have any inhibitory effect on the growth of S. aureus, but did on the contrary enhance it. The stable leukocyte count and adequate function, reflected in steady increase in MPO, C3a and TCC levels during the entire length of the SECC run, indicate that the enhanced S. aureus growth is not a result of NO-effect on the innate immune system but a result of direct NO-actions on the bacteria itself. What is unique for S. aureus, in difference to other bacterial families and Staphylococcus species, is its ability to resist innate immunity and oxidative stress by generation of the enzyme inducible lactate dehydrogenase. Interestingly, NO triggers the generation of this enzyme [102,103]. Therefore, we conclude that supplement of NO to S. aureus that are under oxidative stress will give the bacteria a lactate dehydrogenase-dependent survival advantage (Figure 22). Since we used student T-test for statistical analysis of the results of Study II, one may argue that, in a time-series, this could result in an overestimation of significance in differences. We therefore, after publication of Study II, performed a new statistical analysis of data for S. aureus growth, using repeated measures ANOVA, confirming significant differences between groups in total and over time (p<0.05).
The lack of NO-induced increased inflammatory response in Study II, as determined by increased MPO, C3a and TCC levels, opposes some of our previously reported results [99,104]. One possible explanation may be differences in NO-concentration applied to the SECC circuit; 40 ppm [104] and 80 ppm [99], compared to a mean of 92 ppm in Study II, which raises the question if NO’s pro-or anti-inflammatory effect is dose-related? Depending on experimental conditions, NO can either inhibit or enhance neutrophil activation, in both cases probably acting through cyclic GMP. The explanation for these apparently contradictory findings may be that the effect depends upon the concentration of NO: low concentrations of NO being stimulatory and high concentrations inhibitory [105]. It is possible that, during SECC, NO may over-activate WBC so that they eventually become exhausted. The initial NO effect would be pro-inflammatory but delayed systemic anti-inflammatory. In our SECC model, the exhausted WBC therefore could have limited bactericidal effect on S. aureus. The bacteria may have exerted a direct negative effect on the release of MPO, C3a and TCC, or neutralized these factors, so that no significant differences were seen in Study II between the groups.

Our results in Study IV however showed increased SECC-induced concentrations of MPO, TCC and C3a, supporting our previously reported results [99,104]. The findings in Study I, II and IV indicate that the inflammatory response is not only due to NO effects but also due to SECC effects. In summary, NO affects several subsystems of the homeostasis of the body, and consequently there is potential for a variety of effects in several organ
systems during various pathophysiological conditions. It is known that increased endogenous NO production is associated with organ dysfunction and injury and peroxynitrate with its metabolites seem to be a mediator of tissue injury. We have now confirmed the increased granule release from leukocytes in the NO treated circuit and the lack of protective effect on ROS or interleukin release. This could be one mechanism of action for NO during SECC and may also have clinical significance in clinical CPB. However, these effects were observed in heparinized WBL being ventilated with NO and circulating through an oxygenator at 35°C, and the results cannot be interpreted as NO being detrimental and having no protective effect in a biological system. NO could limit inflammatory tissue injury by preventing leukocyte migration and adherence, decreasing microvascular permeability, inhibiting superoxide production and inactivating superoxide anions in endothelium.

In the clinical setting, use of ECMO to treat patients with acute circulatory and/or respiratory distress is becoming more common [106]. Many of these patients also receive NO to treat concurrent pulmonary hypertension [107]. Serious infections are a major concern since the length of ECMO and NO treatment often extends from several days to weeks [108]. The findings that NO induces more than 6-fold increase in S. aureus growth only after 3 hours certainly adds to this concern.

NO has in studies of sepsis been shown to inhibit leukocyte recruitment to the site of infection [109,110]. Alpha-1-acid glycoprotein, an acute phase protein, isolated from sera of severely septic patients inhibits neutrophil migration in an NO-dependent process [111]. These findings raise additional concerns regarding the use of NO, since it may on one hand promote S. aureus growth and on the other suppress the defense against the bacteria by suppression of leukocyte recruitment.

GTN is an important NO donor widely used in clinical practice for patients with angina pectoris, pulmonary hypertension, and systemic blood pressure control. GTN has been shown to exert a potent inhibitory effect on bacterial growth, including S. aureus, in sodium chloride 0.9%, glucose 5%, ethanol 10%, and Mueller-Hinton broth [112]. Peroxynitrite is a NO-derived oxidant, formed during the reaction between NO and superoxide radicals, acting as a key component of nitrooxidative stress associated with pathophysiological conditions such as inflammatory or cardiovascular disorders. Peroxynitrite demonstrates very strong bactericidal potential, which magnitude depends on many complex factors of kinetics and cellular radical milieu [113].

Effect of GTN on bacterial growth has neither been studied in whole blood nor during ECC.

In Study III, we therefore tested the effect of GTN on S. aureus growth during SECC. We also examined the effect of S. aureus alone and in combination with GTN on activation markers of the innate immune system, includ-
ing CD11b, CD14, CD16, CD32, CD35, CD63, CD64, CD65, and CD66b, during SECC. We showed, in Study III, for the first time, that GTN does not affect S. aureus growth in WBL during SECC. During our experiments, we also screened several important proteins and markers involved in the recruitment and activation of leukocytes in response to S. aureus infection, which allowed us to address the question whether or not GTN addition may affect the activity of the innate immune system.

Study III confirmed that SECC with whole blood infested with S. aureus, induces an activation of both granulocytes and monocytes, as reflected by increases in the majority of measured parameters at most time points. Addition of GTN did not significantly alter expression of the investigated cell surface receptors. A weakness of this study was the low number of experiments performed, and also that the experiments were performed at only one GTN dose. The GTN dose delivered in this study was though correlated to clinically established levels (0–4 mg GTN per mL WBL).

NO has been shown to facilitate the killing of the intracellular pathogen Leishmania donovani, the causative agent of visceral leishmaniasis, by stimulating phagosomal maturation [114].

In a wider perspective, our results may promote further studies in patients with leishmaniasis and/or other bacterial infections. An interesting issue is whether GTN had any effect at all on the innate immune system. Cartwright et al stated that physiologically relevant NO levels did not affect lymphocyte adhesion to endothelium and expression of proteins, such as cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin [115].

ECC always leads to activation of the innate immune system as a part of systemic inflammatory response in which the excessive oxidative stress plays an important role [116].

The interpretation of our results may be complicated by the fact that the ECC-induced activation of different cascade systems alters the pro- and anti-inflammatory balance of the body many times and to various extent during postoperative period. In this process, NO is deeply involved, although its role is still not fully understood. NO may temporarily move this inflammatory balance in beneficial or harmful direction depending on actual status in biochemical and/ or redox milieu [117].

Worth considering is also that the ability of S. aureus to develop resistance to innate immunity by S. aureus adaptive response to nitrosative stress [118]. Also, other recent studies of host–pathogen interaction give us reasons to revise our understanding of the nature of S. aureus infection. The bacteria produce several factors to evade neutrophil response. These include chemotaxis inhibitory protein of staphylococci, staphylococcal complement inhibitor, clumping factor A, and extracellular adherence protein [119,120]. For survival, S. aureus can in fact hide inside neutrophils [121]. The very presence of granulocytes may therefore generate better S. aureus survival. In vitro studies have shown that GTN have a potent but nonspecific im-
munoinhibitory effect on human lymphocyte function by a mechanism other than NO production. GTN induces an antimitogenic effect and inhibits cytokine production and expression, cell-mediated cytotoxicity, and antibody production [122].

Our observed changes were mostly detected at the late stage of SECC, making our findings difficult to relate to settings of more time prolonged ECC such as extracorporeal membrane oxygenation or dialysis. This relating difficulty is also due to the short viability of neutrophils and the fact that in a clinical setting, there is a continuous release of new neutrophils from the bone marrow. In our model, we observed a steadily decreasing number of neutrophils, probably due to a combination of adhesion to the foreign surfaces, mechanical destruction, and apoptosis. What role apoptosis of immune cells play in our settings is unclear. Apoptosis can be initiated through two pathways; the cell kills itself because it senses cell stress or because of signals from other cells. Both pathways induce cell death by activation of caspases and subsequent protein degradation [123]. Our SECC model may have created a stressful environment for the neutrophils, inducing their death and contributing to the increased growth of S. aureus at the late stage of the experiments (Study II and III).

One may question whether our observed changes in Study III are not only due to the time effect of SECC. To clarify this, we performed further experiments in Study IV, with similar settings.

In Study IV, SECC promoted expression of some studied CDs; CD14 and CD35. Moreover, our study for the first time demonstrated that GTN affects SECC induced rise in granulocyte CD32. The reduced expression of CD32 may be due to an internalization of the receptor.

Human IgG has four subclasses, and these have different functions: IgG1 and IgG3 are directed against proteins and are important in bacterial infection and activation of complement. They bind to Fc receptors (FcR) of neutrophils, CD16 and CD32. IgG2 is directed against polysaccharides and binds via CD32 [124]. When bacterial antigens are opsonized by specific IgG, the antibody Fab portions dock onto the bacterial antigen such that the antibody Fc can be presented to neutrophil FcRs. Binding of IgG to FcR allows downstream signalling and activation of neutrophil phagocytosis. Neutrophil activation requires cross-linking of more than one FcR [125].

Being a commonly used NO donor in clinical practice, an immunoinhibitory effect of GTN could have major clinical consequences for postoperative infections requiring prolonged treatment with antibiotics and/or additional surgery. This immunoinhibitory effect was only partly supported by our study since GTN inhibited only granulocyte expression of CD32 but had no effect on the rest of the studied CDs.

Since neutrophil activation requires cross-linking of more than one FcR [125], our observed GTN induced reduction in CD32 expression may have clinical implications due to reduced neutrophil activation and phagocytosis.
Because NO inhibits the expression of endothelial leukocyte adhesion molecules, NO-generating compounds have major therapeutic potential [126]. Tang et al showed in a rat study that GTN reduces neutrophil activation and acute damage in latissimus dorsi muscle graft, possibly by modifying leukocyte activation. MPO levels were markedly lower in GTN treated latissimus dorsi muscle grafts [127]. In an in vivo study with Sprague-Dawley rats, Barry et al showed that GTN prevents neutrophil activation following IR injury. The significant increase in MPO activity and superoxide release produced by IR was prevented by GTN, indicating that GTN administration during the reperfusion phase has the potential to decrease pulmonary microvascular injury [128].

In contrast to Study I, where we observed a pro-inflammatory effect of NO addition during SECC, with increased leukocyte release of MPO and lactoferrin, in Study IV, GTN addition did not have any effect on SECC induced MPO increase. This may be partly explained by the endothelium-depleted SECC model, since the main bioactivation of GTN and nitrite, with further metabolism to NO, is believed to occur in the endothelial vascular layer. The vascular endothelium has been attributed for the main synthesis of NO in the circulation, and red blood cells have been demonstrated to carry a non-functional NOS and, due to their huge haemoglobin content, have been assumed to metabolize large quantities of NO. Recent studies have however shown that red blood cells from humans reversibly bind, transport, and release NO within the cardiovascular system, providing evidence that red blood cells from humans express an active and functional endothelial type NOS [129].

Since it is more complicated to pre-heparinize empty blood bags to avoid the citrate, in Study IV we chose to use both citrate-phosphate-dextrose and heparin. A fair question is how the “multi-cocktail” use of anticoagulants citrate-phosphate-dextrose, heparin and EDTA, may have influenced our results. Philling et al showed that citrate-phosphate-dextrose, but not EDTA and heparin, has an unknown deleterious effect on the ability of monocytes to differentiate into fibrocytes [130]. It is still though unclear if the use of multiple anticoagulants have any influence on leukocyte activation. On the other hand, there are many papers demonstrating ECC induced leukocyte activation.

Activity of antioxidant enzymes of erythrocytes, catalase and glutathione peroxidase, has been shown to decrease after intravenous GTN treatment, resulting in increased levels of ROS [131]. The imbalance between ROS formation and antioxidant defences may result in oxidative stress and subsequent leukocyte apoptosis [132].

Results from a study by Seghaye et al, suggest that NO donor sodium nitroprusside, both in vitro and in vivo, has an inhibiting effect on complement activation initiated by both classic and alternative pathways and that this effect is mediated by NO release [133]. In our study, addition of GTN did
not reduce SECC induced increased levels of TCC and C3a. One explanation may be the lack of endothelium mediated bioactivation of GTN to NO. Also one may speculate whether the GTN induced reduction in CD32 expression may result in a reduced neutrophil activation, since neutrophil activation requires cross-linking of more than one FcR, but with an increased complement activation due to an increase in free circulating IgG. What role apoptosis of immune cells play in our settings is unclear.

Although there were some interesting inter-group differences, with a GTN induced reduction in CD32 expression, a weakness of Study IV may be the low numbers of experiments performed as well as the usage of only one GTN dose, albeit that the GTN dose delivered in this study was chosen in accordance with clinically established levels.
8. Conclusions

Study I
Results indicate that NO addition during SECC is pro-inflammatory by stimulating leukocyte activation and granule release, and has no effect on oxygen free radical production and interleukin release.

Study II
Addition of NO to a SECC system with S. aureus infested whole blood, results in a 6.2 fold bacterial growth in the presence of NO. Results indicate that by stimulating the expression of inducible lactate dehydrogenase, NO may improve S. aureus resistance to oxidative stress, giving the pathogen a survival advantage.

Study III
Results indicate that GTN does not affect S. aureus growth during SECC and has no effect on SECC-induced leukocyte activation.

Study IV
SECC induces increased expression of some leukocyte markers and GTN addition significantly reduces the expression of CD32, the Fcγ-receptor II, partly supporting a possible immunoinhibitory effect of GTN.
9. Limitations and Future Perspectives

The strength of all our four studies is the unique SECC model, allowing studies of the blood and its components, without any influence from other organ systems. This strength is of course also one of the weaknesses of the studies. The body’s organs, biochemical- and immunological systems, and external factors interact in a multi-complex manner, limiting and complicating our understanding of the clinical implications of our findings. Other weaknesses of our studies are the low numbers of experiments performed, the usage of only one NO and GTN dose, and the maintenance of normothermic temperatures in the SECC systems at 35°C.

Further studies are required to increase our understanding concerning nitric oxide’s role in the inflammatory reaction, infection and during extracorporeal circulation. Such studies should include larger numbers of experiments performed, longer SECC times, dose response correlation for NO and GTN, hypo-and hyperthermic SECC temperatures and SECC systems infested with other bacterial pathogens.
Många av de komplikationer som uppstår i samband med hjärtkirurgi beror på de negativa effekterna av extrakorporal cirkulation (ECC). En del av dessa komplikationer härrör från ECC-inducerad aktivering av inflammations- och koagulationssystem. Den inflammatoriska reaktionen kan orsakas av interaktionen av blodkomponenter med luft och de konstgjorda ytorna i ECC systemen, från ämnen som produceras på grund av ischemi-reperfusionsskada i hjärta och lungor, och från ökad frisättning av endotoxiner från ischemiska tarmar. Staphylococcus aureus (S. aureus) är den vanligaste patogenen vid infektioner i luftväggarna, hud och mjukdelar och blodet. Kvävemonoxid (NO) är en gasformad signalmolekyl som är inblandad i många fysiologiska och patologiska processer. NO’s roll vid infektion och inflammation är komplex. NO kan bidra till morbiditet genom att fungera som en vasodilatator, myokardhämmare och cytotoxisk mediator. Å andra sidan kan NO ha en gynnsam roll genom mikrovaskulära, cellskyddande, immunoreglerande, och antimikrobiella egenskaper. En simulerad extrakorporal cirkulationsmodell (SECC) är en sluten krets, innefattande en rollerpump, en oxygenator, en venös reservoar och PVC-rör, där humant helblod cirkuleras. SECC modellen möjliggör studier av blod och blodkomponenter, utan påverkan från andra organsystem.

Studie I. Humant helblod cirkulerades I SECC under 3 timmar, och frisättningen av leukocyt granulae studerades. Resultaten visar att tillsats av NO under SECC verkar pro-inflammatoriskt genom att stimulera leukocytaktivering och frisättning av granulae, men har ingen effekt på varken produktionen av fria syreradikaler eller frisättningen av interleukiner.

Studie II. Vi undersökte effekten av NO på S. aureus tillväxt i helblod under 180 min SECC. Resultaten visar en 6,2-faldig tillväxtökning i närvaro av NO. Resultaten indikerar att NO, genom att stimulera uttrycket av det inducerbara enzymet laktatdehydrogenas, som är specifikt för S. aureus, kan förbättra S. aureus motståndskraft mot oxidativ stress, och därigenom får patogenen en överlevnadsfördel.

Studie III. Vi studerade, i ett in vitro system av SECC, vilken effekt glyceryl trinitrat (GTN) har på S. aureus inducerad leukocytaktivering i helblod, samt effekten av GTN på S. aureus tillväxt. Resultaten indikerar att GTN inte har effekt på varken SECC-inducerad leukocytaktivering eller S. aureus tillväxt under SECC.
Studie IV. Koncentrationen av utvalda leukocyt adhesionsmolekyler, komplementssystemkomponenter och myeloperoxidas i helblod studerades i ett in vitro system av SECC. Resultaten visar att SECC inducerar ökat uttryck av vissa leukocytmarkörer och att tillsats av GTN signifikant minskar uttrycket av leukocyt aktiveringsmarkören CD32, vilket skulle kunna tala för en möjlig immunhämmande effekt av GTN.
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12. References


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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)