From Department of Clinical Sciences, Danderyd Hospital
Karolinska Institutet, Stockholm, Sweden

BIOMARKERS IN ENDOTOXEMIA WITH A SPECIAL INTEREST IN CITRULLINATED HISTONE H3

Sofie Paues Göranson

Karolinska Institutet

Stockholm 2018

Frontpage by Lovisa Baer

Blood drop containing a molecule of H3Cit, leaving (DNA) rings on the water.

Published by Karolinska Institutet.
Printed by Eprint AB 2018

ISBN 978-91-7831-060-9

Biomarkers in endotoxemia with a special interest in citrullinated histone H3
THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Aulan, Danderyds Sjukhus

Fredagen den 1 juni 2018, kl 09.00

Sofie Paues Göranson

Principal Supervisor:
PhD Anne Soop
Karolinska Institutet
Department of Clinical Sciences
Division of Anesthesia and Intensive care

Co-supervisor(s):
Associate Professor Johanna Albert
Karolinska Institutet
Department of Clinical Sciences
Division of Surgery

Professor Claes Frostell
Karolinska Institutet
Department of Clinical Sciences
Division of Anesthesia and Intensive care

PhD Lars Hållström
Karolinska Institutet
Department of Clinical Science Intervention and Technology (CLINTEC)

Opponent:
Professor Hans Hjelmqvist
Örebro University
School of Medical Sciences

Examination Board:
Associate Professor Märta Segerdahl Storck
Karolinska Institutet
Department of Physiology and Pharmacology

Associate Professor Åke Norberg
Karolinska Institutet
Department of Clinical Science Intervention and Technology (CLINTEC)

Associate Professor Sören Berg
Linköping University
Department of Medical and Health Sciences
Division of Cardiovascular Medicine

To my family

“Lyckligtvis är inget omöjligt bara för att det är svårt”.
Gary E. Schwartz

”Det största hindret för nya upptäckter är inte okunnighet – det är illusionen av kunskap”
Daniel J. Boorstin

ABSTRACT

Background: Sepsis with multi-organ failure has an unacceptably high mortality rate of 25-30% despite the use of antibiotics and modern intensive care. Attenuating the multi-organ failure and finding new biomarkers for early detection of septic shock would be of high clinical relevance in order to improve outcome.

Aim: The aim of this thesis was to study if inhaled nitric oxide (iNO) in combination with steroids could attenuate multi-organ failure in a porcine model of endotoxemia. We further aimed to investigate the dynamics of circulating citrullinated histone H3 (H3Cit), a recently proposed biomarker in sepsis, in a human model of endotoxemia. Since microvesicles (MVs) have been shown to be elevated in sepsis, a further objective of this thesis was to investigate whether H3Cit could be detected bound to MVs.

Methods: A randomized controlled trial (RCT) with 30 domestic piglets exposed to lipopolysaccharide (LPS)-alone, LPS + iNO, LPS + IV steroid, LPS + iNO + IV steroid or anesthesia only (Control) was conducted in **paper I**. Various biomarkers were measured at endpoint in order to evaluate organ function after 30 hrs of endotoxic shock. In **paper II**, an ELISA-based assay quantifying plasma H3Cit was developed and methodologically validated in accordance to recommended ELISA validation requirements. This ELISA, as well as a flow cytometric assay quantifying MV-bound H3Cit, was used in plasma samples at baseline and then 2, 4 and 7 hrs after LPS-injection in a placebo controlled RCT including 22 healthy volunteers in **Paper III**.

Results: LPS + iNO+ IV steroid tended to require less norepinephrine and were significantly less acidotic ($p < 0.05$) compared to LPS-only in the porcine model of endotoxemia. No significant differences could, however, be detected in other clinical variables. Circulating H3Cit, quantified by the ELISA assay, which was validated with high specificity, precision and stability, rose significantly after LPS injection in the human model of endotoxemia. Similar elevations were seen when quantifying H3Cit-bearing neutrophil- and platelet derived MVs.

Conclusions: Our data suggest that combined therapy with iNO and IV steroid is at least partially, protective after experimental LPS infusion. We furthermore show that circulating H3Cit, quantified by two distinct methods, is elevated after experimental LPS injection, suggesting that H3Cit may be a novel biomarker even in sepsis. Our data also show that H3Cit can be detected bound to MVs, proposing a novel mechanism by which H3Cit can be transported throughout the vasculature.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following original papers, which will be referred to as Paper I-III:

I. **Paues Goranson S**, Gozdzik W, Harbut P, Ryniak S, Zielinski S, Haegerstrand C. G, Kubler A, Hedenstierna G, Frostell C, Albert J. "*Organ dysfunction among piglets treated with inhaled Nitric Oxide and intravenous hydrocortisone during prolonged endotoxin infusion*." PLoS One. 2014 May 14; 9(5):e96594. doi: 10.1371/journal.pone.0096594. eCollection 2014

II. Thålin C, Daleskog M, **Paues Göranson S**, Schatzberg D, Lasselin J, Laska AC, Kallner A, Helleday T, Wallén H, Demers M. "*Validation of an enzyme-linked immunosorbent assay for the quantification of citrullinated histone H3 as a marker for neutrophil extracellular traps in human plasma*." Immunol Res. 2017 Jun; 65(3):706-712. doi: 10.1007/s12026-017-8905-3

III. **Paues Göranson S**, Thålin C, Lundström A, Hållström L, Lasselin J, Wallén H, Soop A, Mobarrez F. "*Circulating H3Cit is elevated in a human model of endotoxemia and can be detected bound to microvesicles*"
Manucsript submitted.

CONTENTS

LIST OF ABBREVIATIONS

APC	Allophycocyanin
BE	Base Excess
CD	Cluster of Differentiation
cfDNA	Cell-free DNA
CLP	Cecal Ligature and Puncture
CO	Cardiac Output
CRP	C-Reactive Protein
CVP	Central Venous Pressure
DAMPs	Damage-Associated Molecular Patterns
DIC	Disseminated Intravascular Coagulation
ELISA	Enzyme-Linked Immunosorbent Assay
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
H3Cit	Citrullinated histone H3
HES	Hydroxy Ethyl Starch
HMGB1	High Mobility Box Protein 1
Hrs	Hours
I/R	Ischemia/Reperfusion
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-6	Interleukin 6
iNO	Inhaled Nitric Oxide
IV	Intravenous
LPS	Lipopolysaccharide
MAP	Mean Artery Pressure
MOF	Multi-Organ Failure
MPAP	Mean Pulmonary Artery Pressure
MPO	Myeloperoxidase
MVs	Microvesicles
NADPH	Nikotinamid-Adenin-Dinukleotidfosfat Phosphate

NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B-cells
O.D.	Optical Density
p-value	Probability value
PAC	Pulmonary Artery Catheter
PAD4	Peptidyl Arginine Deiminase 4
PAMP	Pathogen-Associated Molecular Patterns
PaO2	Partial Pressure of Oxygen
PBS	Phosfate Buffered Saline
PE	PhycoErythrin
ppm	Parts per million
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PS	Phosfatedylserine
PVRI	Pulmonary Vascular Resistance
RCT	Randomized Control Trial
ROS	Reactive Oxygen Species
RT	Room Temperature
SIRS	Systemic Inflammatory Response Syndrome
SOFA	Systemic Organ Failure Assessment Score
SpO2	Peripheral Oxygen Saturation
SVRI	Systemic Vascular Resitance Index
TLRs	Toll Like Receptors
TNF-α	Tumour Necrosis Factor α
WBC	White Blood Cells
WT	Wild Type

1 INTRODUCTION

1.1 MEDICAL PROBLEM/BACKGROUND

The mortality in severe sepsis and septic shock is still unacceptably high, although it tends to decrease from around 30-80% to 20-30% through improved bundles of early interventions and advances in training and surveillance [2-6]. Patients with septic shock and multi-organ failure (MOF) account for the highest mortality [4, 7]. Clearly, antibiotics are of vital importance in order to eliminate bacteria that constitute the initial cause of septic shock. Nevertheless, up to date there are no documented treatments in attenuating MOF while the antibiotics exerts its full effect. Therefore, treatments in order to reduce MOF are stressed in order to decrease mortality resulting from sepsis.

The fundamental theory that sepsis is an effect of uncontrolled inflammation has lately been disputed. Instead, the dynamics of the immune profile have been urged to be of importance, going from a proinflammatory state with e.g. a massive cytokine response initially, to a rather immunosuppressed condition at a later stage [8-11]. Hence, not considering these different immune phases could be one cause of some disappointing results historically in trying to inhibit the immune response [8], by e.g. an early cytokine activation administering anti-Tumour Necrosis Factor α (anti-TNFα) or anti-Interleukin 1 (anti-IL1) [12], as well as corticosteroids of 30 mg/kg [13] and anti- endotoxin antibodies [14].

Consequently, it is of vital importance to understand the pathogeneses of sepsis, which can lead us towards useful biomarkers and possible targeted therapies that could attenuate the multi-organ failure connected with septic shock. Further, more useful future biomarkers should enable the differentiation of patients at risk for more severe infections and could also be of help in initiating correct treatment as well as facilitating prediction of outcome.

A biomarker can be defined as a characteristic by which a pathophysiologic process might be identified [15]. Up to date several different biomarkers for sepsis have been studied but their clinical effectiveness in predicting sepsis remains elusive [15, 16]. The currently updated guidelines from “survival sepsis campaign” [17] discuss minor use of only one biomarker, procalcitonin, for help in guiding antibiotic treatment, even though both elevated C Reactive Protein (CRP) and in particular lactate, are widely used tools in order to identify and predict sepsis diagnosis and its outcome in clinical practice [1, 18, 19]. Hence, the research about biomarkers in sepsis is still at its beginning and should be warranted in order to ameliorate the outcome of this lethal disease.

1.2 DEFINITION OF SEPSIS

1.2.1 Classic definitions

For many years, sepsis has been considered a stepwise progression reflecting an exaggerated host response to inflammation [20]. The proposed definitions are listed below:

1.2.1.1 Systemic Inflammatory Response Syndrome

The first stage is the systemic inflammatory response syndrome (SIRS). SIRS is considered an inflammatory condition that may harm the patient. Triggers to SIRS can be infection, trauma, pancreatitis, burns and different kinds of shock.

SIRS is apparent when > 2 of the signs listed below are apparent:

- Temperature > 38 or < 36°C,
- Heart rate > 90 beats per minute
- Respiratory rate > 20 per minute / pCO2 < 4,2 kPa,
- White Blood Cell (WBC) count > 12 x10^9 or < 4 x 10^9/L or > $10^{\%}$ immature neutrophils "bands"/forms

1.2.1.2 Sepsis

Sepsis is defined as simultaneous occurrence of SIRS + infection

1.2.1.3 Severe sepsis

Sepsis+ organ dysfunction (e.g. signs of respiratory failure, hypotension and hypoperfusion).

1.2.1.4 Septic shock

When severe sepsis goes with refractory hypotension, meaning an inadequate response to "adequate" amount of fluids, septic shock is apparent.

In the revised definitions of sepsis from 2001, Levy et al concluded that even if the traditional classification based on SIRS might remain helpful to clinicians and researchers, the concept is overly sensitive and non-specific. Therefore, the authors presented an expanded list of symptoms and signs of sepsis (e.g. oliguria, elevated creatinine, lactate > 1) and also concluded that "the use of biomarkers for diagnosing sepsis is premature" [19].

1.2.2 New proposal for the sepsis definition

Recently the SIRS-based definitions defined by Bone et al have been challenged [1]. The proposition called "Sepsis-3" is a new definition of sepsis: *"Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection".* The diagnostic criteria of Sepsis-3 is based on the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) [21], composed of a scoring system from six organ functions (cardiovascular, respiratory, hepatic, renal, coagulation and neurological), graded in relation to the degree of the dysfunction. By this definition, the notion of severe sepsis is redundant. Even the term SIRS is obliterated due to its unspecific nature. In Sweden, a consensus group recently decided to use the new definitions and diagnostic criteria for sepsis as well as for septic shock. However the use of the new screening tool, "quick-SOFA", proposed by Sepsis-3, will not be applied in Sweden, until prospectively validated [22].

Definitions of sepsis and septic shock according to Sepsis-3 [1]

- **Sepsis:** Infection+ acute change in total SOFA score >= 2 points.
- **Septic shock:** Infection + persisting hypotension requiring vasopressors to maintain MAP > 65 mm Hg + serum lactate level > 2 mmol/L (18 mg/dL) despite adequate volume resuscitation.

1.3 THE IMMUNE SYSTEM

The immune system is a complex system of biological processes and structures within an organism that defends it against disease. In order to function accurately, the immune system must discover a variety of pathogens and differentiate them from endogenous healthy tissue.

The immune system can be sub-classified or sub-divided into the ***innate-*** (unspecific/native) and the ***adaptive*** (specific) immune system that is acquired later in life, as opposed to the innate immunity [23]. Included in the adaptive immune system is a so-called, immunological memory [24]. It comprises antibodies from B-lymphocytes that eliminate or neutralize microbial toxins and microbes, existing outside the host cells, constituting the humoral immunity. Furthermore, T-cells that constitute another part of the adaptive immune system, act by inducing apoptosis for infected cells and is called “cell mediated immunity”.

This thesis will however, focus mainly on the innate immune system, with a special interest in a recently described subgroup of components called neutrophil extracellular traps (NETs).

1.3.1 The innate immune system

The innate immune system exists from birth and serves as a first line defense when the body is infected by a pathogen. Unlike the adaptive immune system, it cannot adapt to a certain pathogen. Nevertheless, the innate system mobilizes quicker than the adaptive defense.

The innate immune system comprises of neutrophils and monocytes, acting by phagocytosis (ingestion) and destruction of microbes; natural killer (NK) cells that kill pathogen-infected cells; endothelial cells; monocytes that together with dendritic cells serve as antigen-presenting cells, thereby stimulating the subsequent adaptive immune response [23]. When a monocyte exits from the vascular space it develops into a macrophage. Macrophages phagocyte invading pathogens, but can also secrete cytokines, which recruit additional immune cells to the infected area [23].

An overview of the innate- and adaptive immune system is presented in figure 1.

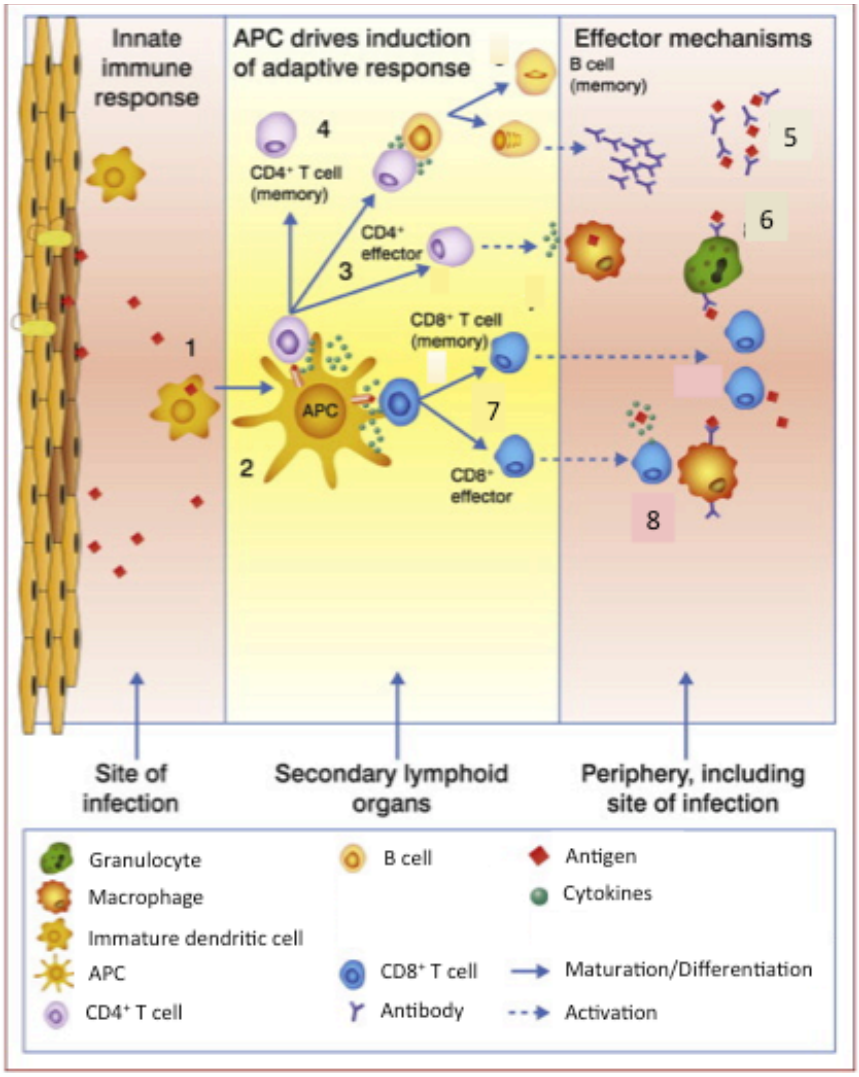

Figure 1. Overview of the human immune response. *Reprinted from "Understanding Modern Vaccines: Perspectives in Vaccinology" Leo et al (2011); J. Pervac; 1(1) 25-59 with permission by Elsevier through CC BY-NC-ND 3.0 license. © 2011 Elsevier B.V.*

The innate immune system, the first line of defense, comprises of several immune cells. Granulocytes are subdivided into neutrophils, basophiles and eosinophils. **1**) After activation of the innate immune system, granulocytes together with monocytes and their extravascular form, macrophages, ingest/phagocyte pathogen-infected cells, which are subsequently killed by Natural killer cells (NK). **2)** Dendritic cells mature into antigen-presenting cells (APC) in presence of a pathogen and serve as an important link in triggering the adaptive immune system that are developed later in life. Activation of the adaptive immune system includes **3)** secretion of cytokines by the $CD4^+$ T-helper cells that also **4)** stimulate B-cells to secrete antibodies. **5)** Antibodies can neutralize antigens and **6)** enhance functionality in innate immune cells. Further, **7)** $CD8^+$cytotoxic T-cells are activated **8)** inducing apoptosis in infected cells by. All together, these actions lead to efficient clearance of pathogen by among other things, production of anti-bodies.

APC = Antigen presenting cell; CD = cluster of differentiation

1.3.1.1 Activation of the innate immune system by PAMPs and DAMPs

The ability of the macrophage to promptly fight infections is due to receptors in their cell membranes, recognizing foreign components from e.g. bacteria. These pathogen components are often called "pathogen-associated molecular patterns" (**PAMPs**) [25]. Lipopolysaccharide (LPS) incorporated in the bacterial wall of Escherichia coli (E. coli) is an example of a PAMP. PAMPs also include foreign molecules released from bacterial lysis, such as bacterial DNA fragments and heat-shock proteins.

The activation of the innate immune defense could also arise after recognition of *endogenous* molecules released during cell death or cell injury, known as damage-associated molecular patterns (**DAMP**s). One example of DAMPs is High Mobility Box Protein 1 (HMGB1). HMGB1 is a nuclear protein that translocates from the nucleus to the extracellular space in response to inflammation, thereby executing downstream cytokine stimulation [26].

1.3.1.2 Toll-like receptors

Receptors binding/recognizing DAMPs and PAMPs are called pattern recognition receptors and include toll-like receptors (TLRs) [27-29]. In mice and humans there are 10 known TLRs [29]. Stimulation of TLR leads to induction of inflammatory cytokines and antimicrobial genes. Furthermore, activation of TLRs generates dendritic cell maturation that results in increased antigen-presenting capacity. Hence, microbial detection by TLRs not only triggers the innate immune system, but also helps guiding the adaptive immune responses. Andonegui et al [30] showed that platelets present the TLR4, thus playing a role in the inflammatory response. Moreover, the cytokine response generated from translocation of HMGB1 to the extracellular space, is mediated by the TLR4 [26].

1.3.1.3 DAMPs as biomarkers

Several DAMPs have been evaluated as biomarkers in sepsis. In addition to HMGB1, cell-free DNA (cfDNA) has been proposed as a potential biomarker of use. CfDNA is defined as DNA strands, normally enclosed within the cell nucleus, circulating freely in the blood stream. Indeed cfDNA has been shown to be elevated in e.g. apoptosis and necrosis [31, 32]. Moreover cfDNA has been used as an indirect marker of another molecule of the innate immune system: Neutrophil Extra Cellular Traps (NETs).

PAMPs: Patterns on ***pathogen*** recognized by the immune system (e.g. LPS, bacterial DNA fragments)

DAMPs: ***Endogenous*** molecules released (e.g. cfDNA, HMGB-1)

1.3.2 Neutrophil extracellular traps (NETs)

Neutrophils are the most abundant white blood cells, and their antimicrobial activities by phagocytosis and degranulation are well defined. Interestingly, a third antimicrobial mechanism was described just over a decade ago [33], in which the neutrophils release their nuclear content in the form of web-like structures consisting of DNA strands, coated with histones and granular proteases, referred to as neutrophil extracellular traps, NETs.

The mechanisms by which neutrophils release NETs are not fully understood, but the assembly of Nikotinamid-Adenin-Dinukleotidfosfat phosphate (NADPH) oxidase upon neutrophil activation, leading to the production of reactive oxygen species (ROS) and the activation of the enzyme peptidylarginine deiminase 4 (PAD4) has been shown to play a central role. Upon activation, PAD4 enters the nucleus and converts positively charged arginine residues to uncharged citrulline residues (i.e. citrullination) on histone H3 (referred to as H3Cit), thereby reducing its positive charge. The production of ROS also triggers the leakage of granular proteases, such as neutrophil elastase (NE) and myeloperoxidase (MPO), which move to the nucleus and cleave histones. Citrullination and cleavage of histones result in chromatin decondensation, the initial step of NETosis. Decondensated chromatin coated with the antimicrobial granular proteases (i.e. NETs), are subsequently extruded into the extracellular space, where they are shown to kill microbes both by physical entrapment, as well as by the high concentration of antimicrobial proteases [33]. ROS-independent NETosis has also been described [34].

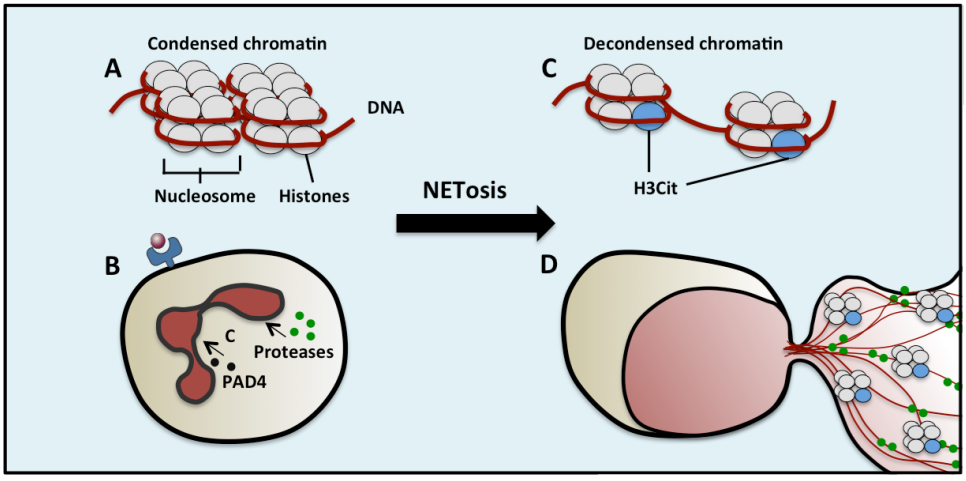

Figure 2. NETosis. *Reproduced with permission from Charlotte Thålin, MD, PhD.*

A) Nucleosomes are tightly packed DNA-strands coiled around a core of 8 histones, and organized into condensed chromatin in the nucleus. **B)** Peptidylarginine deaminase 4 (PAD4) is an enzyme primarily expressed in neutrophils. Upon activation of the neutrophil, PAD4 and granular proteases initiate NETosis, by translocating into the nucleus where **C)** PAD4 citrullinates the arginine residue of histone H3 and the proteases cleave the histones, both actions resulting in chromatin decondensation. **D)** Finally, the decondensed chromatin, coated with antimicrobial proteases, is extruded into the extracellular space.

1.3.2.1 Suicidal and vital NETosis

The schematic in figure 2 illustrates the ROS-dependent and "lytic" or "suicidal" NETosis. This process, necessitates lytic cell death of the neutrophil and takes up to 2-3 hours if the neutrophil is directly stimulated by the pathogen [35].

A different form of NETosis, "vital NETosis" has also been reported [36]. Vital NETosis involves budding of microvesicles transported into the extracellular space, where they rupture and release NETs. This process takes 5-60 min, does not require neutrophil lysis and is ROS-independent. Notably, vital NETosis preserves neutrophil function including the recruitment cascade, phagocytosis, chemotaxis and microbial killing [36].

1.3.2.2 Triggers for NETosis

NETosis can be triggered in several ways, e.g. by intracellular oxidative stress, Staphylococcus aureus [36, 37], fungi and E. coli, and LPS [38]. However, only a portion of a neutrophil population will release NETs in response to stimuli [39]. Platelets also have a role in NET formation by binding LPS via TLR4 on their surface and presenting LPS to the neutrophil, initiating NETosis in the smallest vessels of lung and liver. NETosis induced by platelet-LPS interaction only takes minutes [40].

1.3.2.3 Clinical role of NETs

Neutrophils use three main strategies in the defense towards microbes: phagocytosis (within 10 min and pose no harm to the host), degranulation (within 30 min, certain damage to the host), and NET formation (within 10 min-3 hrs depending on NETosis trigger.) NET are a strategy to kill and trap bacteria within the NET, thus trying to shelter the host from neutrophil granualae that could cause damage [35].

Elevated plasma concentrations of NETs have been noted in both endotoxemia [41] and sepsis [42], where they are thought to play an important role in the early pathogen defense, possibly inhibiting dissemination of local infection. On the other hand, excessive formation and insufficient regulation of NETs may be harmful to the host. For example, NETs could promote inflammation and occlude capillaries which impair the micro-circulation [43]. Histones released from neutrophils have also been shown to harm organs/tissues [42, 44]. Indeed, high levels of NETs seem to be correlated to the severity of sepsis and multi-organ failure [45]. Therefore, it has been discussed about the possible use of NETs, or its components, as predictors of multi-organ failure and mortality in sepsis. Up to date, it is not known at what point NETs become detrimental, but the fine-tuning of NETosis throughout the septic immune response, could be a focus of development for new NET-targeted sepsis-therapies.

Even though NETs are implicated in the defense of microbes and consequently have a role in infection, NETs have also been associated with several non-infectious and sterile conditions such as thrombosis [46], systemic lupus erythematous [47], acute lung injury [48, 49], small vessel vasculitis [50], diabetes [51] and cystic fibrosis [52]. Recently, NETs have also been

proposed to play a role in tumor progression [53], metastatic spread [54, 55] and cancer-associated thrombosis [56, 57]. Notably, NETs have been shown to accumulate in peripheral blood vessels and impair vascular function in tumor-bearing mice [58] and markers of NETs have been detected in widespread microthrombi in cancer patients, possibly contributing to MOF, as has been shown in sepsis [45].

1.3.2.4 Methods of measuring NETs

NETs can be visualized by histology utilizing light- and electron microscopy [59]. However, microscopy comes with two major limitations: the lack of objectivity and the lack of quantification [60]. Quantification of surrogate NET markers, such as the NET-associated granular proteases NE and MPO and cfDNA, by commercially available enzyme-linked immunosorbent assays (ELISAs) have therefore been used to measure the levels of NETs in plasma. Yet, results from the methods mentioned may have to be interpreted with caution since cfDNA can arise from events other than NETosis [61]. Also, MPO and NE can exist from neutrophil activation not resulting in NETosis [62, 63]. In the context of biomarkers in sepsis, increasing attention has revolved around the NET-specific component citrullinated histone H3 (H3Cit), being a more specific marker for NETosis than e.g. cfDNA or the granular proteases. However, until recently, authors have detected H3Cit mainly by Western blot [64] in lack of a validated ELISA for the quantification of plasma H3Cit.

1.3.3 Microvesicles

In addition to NETs, activated neutrophils can also generate so called microvesicles (MVs), also known as microparticles.

1.3.3.1 Definition

MVs are commonly described as a heterogeneous population of small blebs with a diameter of 100 to 1000 nm [65]. These phospholipid vesicles are shed from the plasma membrane of not only neutrophils but from all cell types such as other leukocytes, platelets and endothelial cells during activation or cell death (apoptosis) [66], as illustrated in figure 3.

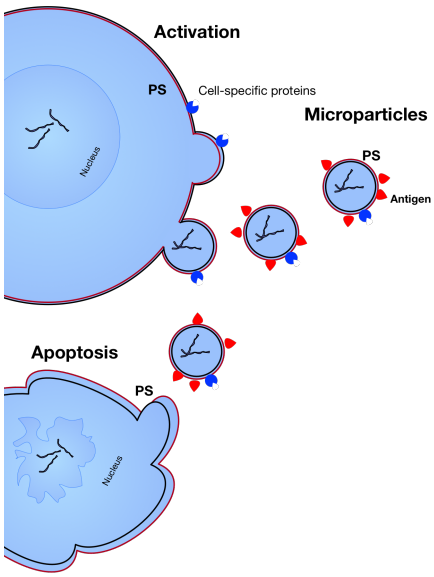

Figure 3. Release of microvesicles (MVs) from an activated or apoptotic cell. *Reproduced with permission from Fariborz Mobarrez, PhD.*

Upon activation or apoptosis, small blebs called MVs are budded from the plasma membrane from cells such as leukocytes and platelets. The cell membrane of the MVs is rich in phosfatedylserine [65, 66], which make them negatively charged. MVs also carry antigens from the host cell, thereby enabling dissemination to other parts of the body.

PS= Phosfatedylserine

1.3.3.2 Function of microvesicles

MVs can carry antigens and receptors from their cell of origin, thus transferring these surface-signaling molecules to other cells of different origin, initiating intracellular signaling pathways.

Increased levels of MVs have been observed in various medical conditions, such as arterial thrombosis [66], diabetes [67], subarachnoid hemorrhage [68] and also in schizophrenia [69]. MVs are elevated in sepsis and may have deleterious effects on a number of tissues and may also contribute to organ dysfunction in septic shock [70, 71]. Furthermore, endothelial MVs have been proposed as a predictive biomarker of early septic shock-provoked disseminated intravascular coagulation (DIC) [72], even though the main part of circulating MVs in sepsis is platelet-derived [73]. Nevertheless, the use of MVs as a diagnostic and prognostic biomarker in sepsis remains elusive. MVs contribute to sepsis pathology in several ways, which is summarized in figure 4.

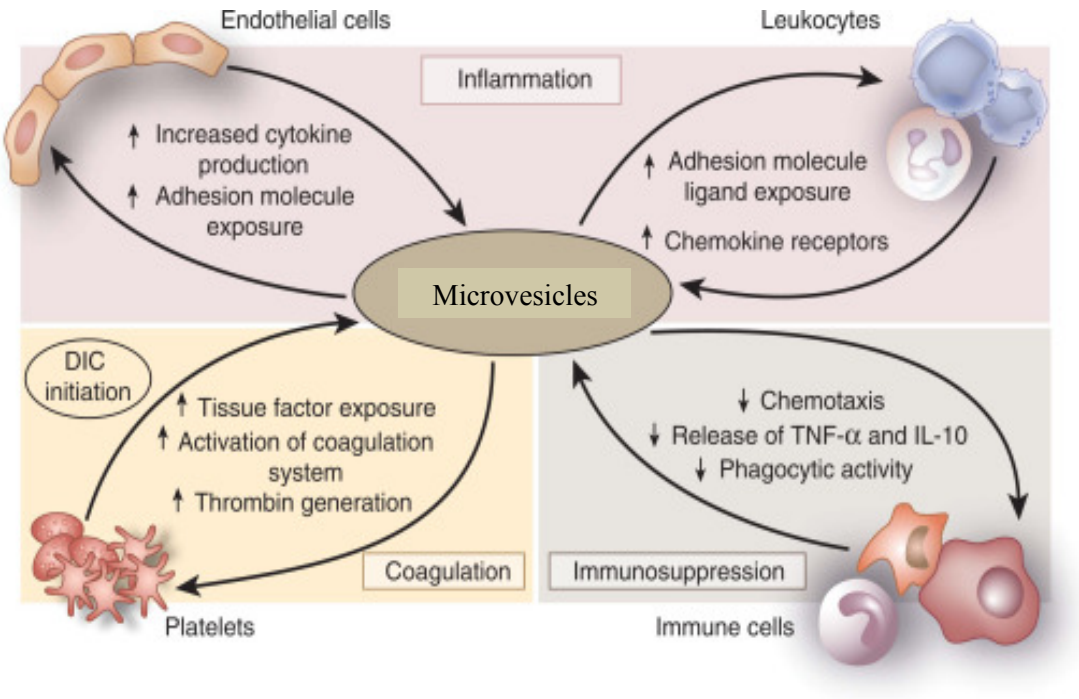

Figure 4. Schematic of MV-actions in sepsis. *Reprinted from "Microparticles: markers and mediators of sepsis-induced microvascular dysfunction, immunosuppression, and AKI." Souza, A. C., et al. (2015). Kidney Int. 87(6): 1100-1108, Copyright (2015), with permission from Elsevier.*

In response to microvascular injury associated to sepsis, immune cells such as platelets, endothel cells and neutrophils release mirovesicles (MVs). MVs can be both proinflammatory, immunosuppressive, as well as protrombothic that further enhance the microvascular injury.

DIC = Disseminated intravascular coagulation; TNF-α = Tumor necrosis factor α, IL-10 = Interleukin 10.

Additionally, MVs are elevated after endotoxin injection and can expose the pro-inflammatory molecule HMGB1 and other cytokines [74]. MVs can also increase in blood during smoking, showing that MVs are sensitive for inflammation [75].

1.3.3.3 Methods of measuring microvesicles

There are several methods of measuring MVs. Flow cytometry is the most commonly used method, thus enabling phenotyping of the MVs. To determine the origin of the MVs, samples are labeled with monoclonal antibodies against known cell-markers as illustrated in table 1 below.

Antigen	**Parent cell**	**Alternative Name**
CD45	Leukocyte	
MPO/66b	Neutrophils	
CD42a/41/61	Platelet	GPIX
CD14	Monocyte	
CD62E	Endothelial cell	E-selectin

Table 1. Antigens/cell-markers of different cell-types

In this thesis CD42a (platelet marker), MPO and CD66b (neutrophil markers) have been used in order to phenotype MVs.

1.4 LINK BETWEEN INFLAMMATION, COAGULATION AND MOF

The fact that both NETs and MVs have been reported to be pro- coagulant [76, 77], illustrates the close link between inflammation and coagulation. Furthermore, platelets have been shown to have a prominent role in inflammation, triggering both the innate and adaptive immune system [78]. In a complex chain of events the imbalance between coagulation and

anti-coagulation, together with inflammation result in organ damage. Figure 5 illustrate the link between inflammation, coagulation and the resulting organ damage as a schematic.

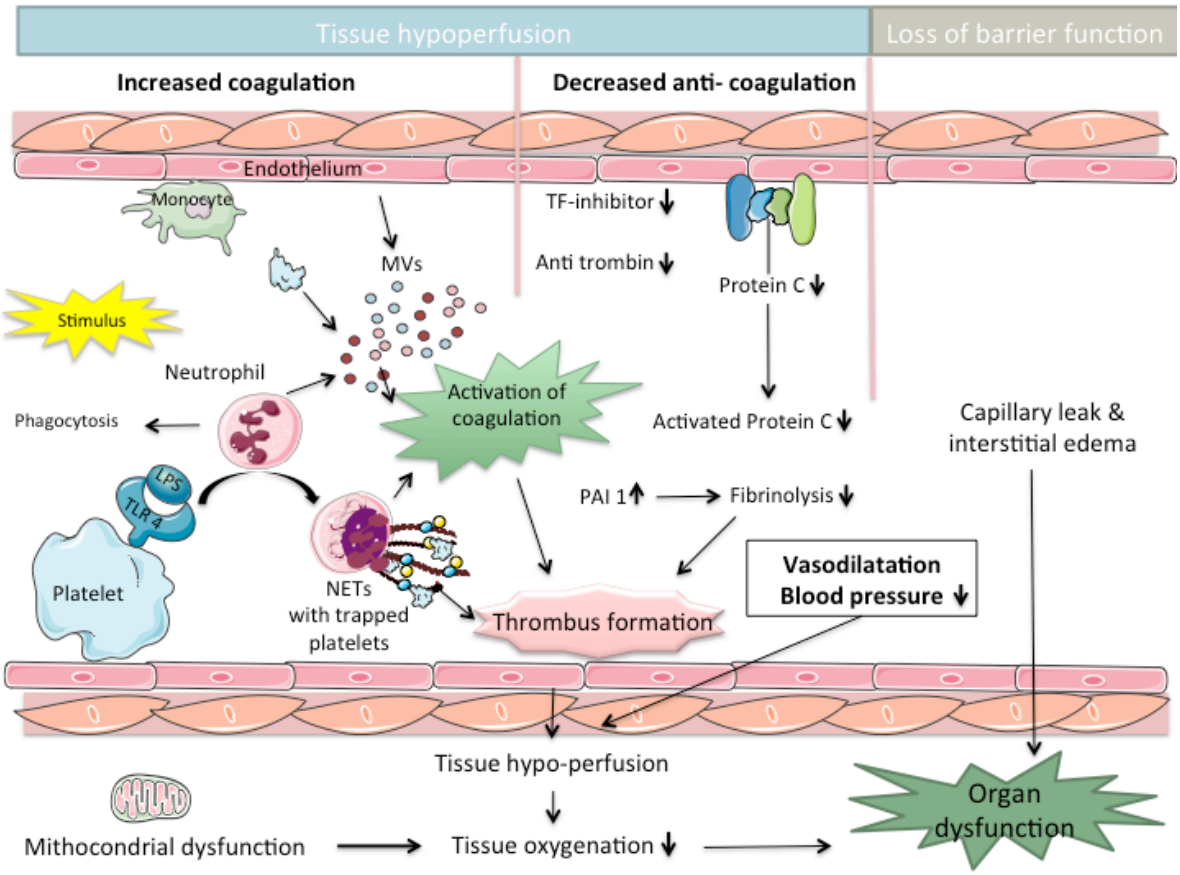

Figure 5. Link between inflammation, coagulation and organ dysfunction.

Severe sepsis is associated with microvascular thrombosis and micro-vascular dysfunction. DAMP molecules e.g. LPS or external pathogens, trigger the immune system. Platelets activate and induce neutrophils to NETosis by presenting LPS through their TLR4 [40]. Circulating platelets are subsequently trapped in the NETs, which promote thrombus formation. NETs can also activate coagulation by the intrinsic pathway [79]. Activated immune cells, like neutrophils, platelets and endothelial cells, further release MVs that also have pro- coagulant activity [70]. Together with an impaired anti-coagulation, resulting from decreased levels of antithrombin, tissue factor pathway inhibitor and activated protein C, in addition to impaired fibrinolysis, due to an increased level of plasminogen activator inhibitor type 1 (PAI-1), thrombus formation increases. This results in tissue hypoperfusion, which is aggravated by vasodilatation, inducing low blood pressure. Together with an increased vascular leakage that produces tissue edema and a mitochondrial dysfunction, this tissue hypoperfusion result in organ dysfunction [6].

TLR4 = Toll-like receptor 4; LPS = Lipopolysaccharide; NETs = Neutrophil extracellular traps; MVs = Microvesicles; TF-inhibitor = Tissue Factor inhibitor; PAI-1 = Plasmin activator inhibitor type 1.

1.5 ENDOTOXIN STIMULATION AS EXPERIMENTAL INFLAMMATION

Designing a clinical sepsis survey presents great challenge. This is due firstly, to that patients present at different stages of sepsis and secondly because of the diversity of microbiological patterns, as well as the heterogeneity among the patients. Therefore, experimental research is essential in order to increase the understanding of sepsis pathogenesis in a standardized manner. Numerous animal models using several species including rats, mice, rabbits, dogs, pigs and sheep have been described. Models include soft tissue infection, pneumonia model, meningitis model and intravascular infusion of live bacteria or endotoxin [80]. Endotoxin models is the focus within this thesis.

1.5.1 Immune response following endotoxin stimulation

Endotoxin/bacterial lipopolysaccharide (LPS) is incorporated in the cell membrane of most gram-negative bacteria, serving as a PAMP/signal flag, triggering the host-immune system and often induce the immunological septic reaction. The recognition of LPS by TLR4 on macrophages, induces the production of cytokines and other pro-inflammatory mediators through the Nuclear Factor Kappa-light-chain-enhancer of activated B-cells (NF κβ) pathway. This so called “stress-responsive” transcription factor, regulates the gene expression of different cytokines. The activation results in the synthesis and release of various cytokines considered characteristic for sepsis and the systemic inflammatory response syndrome (SIRS). NF κβ may also be induced by cytokines, through their respective receptors on the cell membrane [81].

Endotoxin is manufactured from the E. coli bacteria. It is used in the experimental setting to activate the immune system. Not containing the microorganism, it is a reliable, reproducible and widely used experimental method in both animals [82, 83] and humans [84-87].

The inflammatory response, following endotoxin administration, depends on several factors, including dose and route of administration as well as host factors. Symptoms increase with a higher dose for all species and cytokine profile changes when a continuous infusion of LPS is used compared to when a single intravenous injection is administered as illustrated in figure 6 [88]. Further, pigs have been reported more resistant to LPS compared to both primates and humans [89].

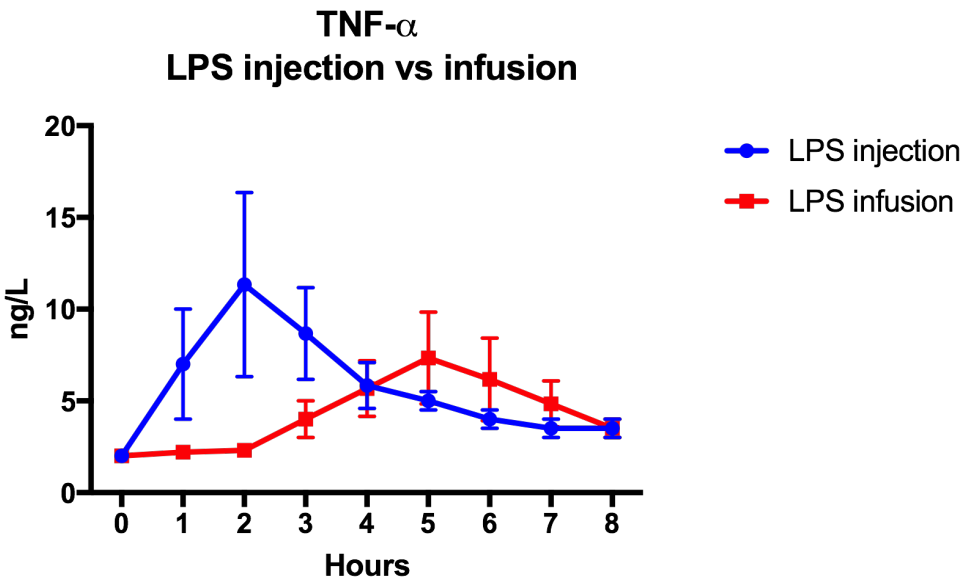

Figure 6. Plasma TNF-α after LPS (0.3 ng/kg), given either as an IV bolus injection or a 4 hrs IV infusion in healthy volunteers. *Inspired by data and modified from Taudorf et al. Clin. Vaccine Immunol, 2007, 14(3), 250-255 [88] with permission by the Creative Commons Attribution 4.0 International license.*

TNF-α levels peaked earlier, at 2 hrs after LPS injection compared to the 4 hr IV infusion that peaked at 5 hrs post start.

TNF-α = Tumor necrosis factor α; LPS = Lipopolysaccharide; IV = intravenous, ng/L = nanogram per liter

1.5.2 Animal models

In order to create an experimental septic shock-like state, higher doses of endotoxin have been utilized in pigs [83], mice [90] and baboons [91]. In order to obtain a sustained immunological reaction, endotoxin can be administered by infusion during the entire or part of the study time. In µg/kg doses of endotoxin, the LPS reaction is sudden and vivid in pigs, producing a decrease in systemic blood pressure and a transient increase in mean pulmonary pressure (MPAP) [83].

1.5.3 Experience in humans

Very low doses (< 1 ng/kg body weight) of endotoxin result in minimal changes in clinical signs, low levels of cytokines and short periods of depressed mood, reduced appetite, fatigue and cognitive impairment. Endotoxin doses of 2-4 ng/kg, elicits a series of symptoms and clinical signs that may show the initial phase of the immune activation [92].

1.5.3.1 Endotoxin (2-4 ng/kg) effects on immune cells and symptoms

Clinical symptoms, like headache, usually occur 1 hr post LPS injection. Later **increased body temperature, heart rate, myalgia, and nausea** may appear. Recovery from these symptoms occurs after 8 to 12 hrs post LPS injection. Immediate **release of cytokines** [93, 94] is followed by **vasoconstriction** [91]. Additionally, LPS activation induces a **drop in white blood count (WBC) at 1hr**, caused by neutrophil depletion during the first hour [87, 93, 95]. The initial drop in circulating neutrophils has been explained as probable increased cell margination along the endothelium, due to both LPS and cytokine-mediated up-regulation of vascular adhesion factors [87].

The leukopenia is followed by a leukocytosis, peaking at 8 hrs post LPS injection, before returning to baseline [84]. The leukocytosis may be explained by cytokines (e.g. IL 8) promoting the mobilization of neutrophils from bone marrow stores [87].

Further, **Microvesicles** derived from platelets ($CD42a^+$), monocytes ($CD14^+$) and endothelial cells ($CD62E^+$) significantly increased after a bolus injection of LPS despite administration of hydrocortisone in human volunteers [74].

1.5.3.2 Endotoxin (2-4 ng/kg) effects on coagulation

After endotoxin, an **early activation of the fibrinolytic system occurs**, which is then followed by a later and more prolonged **activation of the coagulation** [87]. Moreover, a moderate decline in **platelets** is a known effect of LPS stimulation [94]. The reason for this decline seems to be unknown.

1.5.4 Difference between endotoxin stimulation and sepsis

The endotoxin model has been criticized of being an artificial model not reflecting the clinical reality. Hence, since an actual bacteria is not involved, the reaction from LPS cannot be interpreted as sepsis. Moreover, different microbial agents can modulate the immune response differently [96]. Indeed, since LPS is just being one part of the gram-negative bacteria, it has been suggested that other parts of the cell membrane might be of importance in the sepsis reaction [80]. Further, in endotoxemia a more rapid increase in proinflammatory cytokines (e.g. IL-1β, IL-6 and TNF-α) has been reported, compared to the cytokine response in sepsis [97]. Therefore, in order to overcome these issues other animal models including cecal ligature and puncture (CLP) have been described [97].

1.6 SOME TREATMENTS AIMING TO ATTENUATE MOF

The most important measures in order to ameliorate the outcome in sepsis include early interventions like antibiotics and supporting vital functions, like normalized blood pressure by fluid resuscitation and inotropic drugs [17]. However, down below are presented some other treatments that have been used in sepsis therapy over the years and that will be considered within this thesis.

1.6.1 Inhaled Nitric Oxide

Inhaled nitric oxide (iNO) attenuates pulmonary hypertension by selective relaxation of vascular smooth muscle cells in ventilated lung regions, thereby improving arterial oxygenation [98, 99]. Lately, it has been accepted that iNO also may have systemic effects [100, 101]. Neither the extent of iNO's extra-pulmonary effects, nor the mechanisms, are entirely known. Though, it has been suggested that iNO is transported systemically by so called nitrosothiols. Nitrosothiols constitute of thiol groups attached to proteins and have been suggested to serve as nitric oxide (NO) donors outside the lung, through nitrite metabolites [102]. NO has also been shown to have anti-inflammatory effects, by inhibiting the expression of interleukins, adhesion molecules, cytokines and other inflammatory components like neutrophil infiltration [102-106].

In summary, these data suggest that iNO could possibly be a novel option in treating diseases characterized by systemic endothelial dysfunction.

1.6.2 Glucocorticosteroid

Glucocorticosteroid has well known anti-inflammatory effects, both by repressing the transcription factor AP-1, as well as inhibiting the transcription of the NF κβ- pathway [107]. In addition to the immunomodulatory effects, steroid administration may also have hemodynamic impact given in a low dose i.e. 200 to 300 mg/day of hydrocortisone or equivalent [107]. Some septic patients suffering from adrenal insufficiency, which makes them less sensitive to norepinephrine, have been reported to get an improved vasopressor response by hydrocortisone [108].

Conflicting evidence have been reported about steroid use in septic shock. Two systematic reviews showed an improved septic shock reversal by use of a low-dose steroid treatment [109, 110]. However, while one of the aforementioned studies did find a decrease in mortality by prolonged low-dose treatment of steroid [109], the other did not [110]. A more recent study showed that patients with septic shock randomized to 200 mg of hydrocortisone/day for 7 days had a quicker resolution of shock (3 vs. 4 days), a shorter duration of the initial mechanical ventilation (6 days vs. 7 days) and required less blood transfusion (37.0% vs. 41.7%) compared to the placebo group [111]. Neither this study, nor a large European multicenter trial (CORTICUS) [112] could, however, prove any reduction in mortality in the hydrocortisone group.

Still, the survival sepsis campaign [17] suggests a low dose glucocorticoid, intravenously (IV) of (< 200 mg/day) for patients with vasopressor resistant septic shock, in the absence of hypovolemia.

1.6.3 Combination of iNO and glucocorticosteroid

Hence, iNO and corticosteroid administration have both been shown to modulate the inflammatory process by among other actions, inhibition of NF-κB activation [105-107].

Da et al. [83] showed that iNO combined with intravenous (IV) hydrocortisone did have synergistic effects in organ protection. In a six-hour porcine endotoxemia model, administration of IV hydrocortisone simultaneously with iNO

30 ppm almost preserved normal histology of the kidneys, liver and lungs, changed the pathophysiological reaction in a beneficial manner and blunted the inflammatory response. Renal, hepatic and pulmonary glucocorticoid receptors were down regulated by endotoxin infusion and subsequently up regulated by administration of iNO started after 3 hrs of endotoxin infusion.

Conversely, Hållstrom et al. found no difference in cytokine expression between LPS only and LPS + iNO + IV steroid in a human endotoxin model (LPS 2 ng/kg) [86].

2 AIMS

The overall aim of this thesis was to explore possible attenuating pathways in multi-organ failure after an endotoxin challenge in pigs and to better understand the immune response associated with endotoxemia.

More specifically, the studies aimed to:

- Report **the effect on the multi-organ failure** associated with prolonged endotoxemia (30 hrs) in pigs when treated **with inhaled Nitric Oxide** (iNO) 30 ppm, **in combination with** intravenous **steroid** (hydrocortisone, 75 mg x 3). (**Paper I**)

- To methodologically **validate a new** enzyme-linked immunosorbent assay **(ELISA) in order to quantify** the levels of the potential sepsis marker citrullinated histone H3 (**H3Cit**) in human plasma. (**Paper II**)

- To **assess the effect of endotoxin on circulating H3Cit**, in a human model of endotoxemia and to investigate a possible presence and cellular origin of H3Cit-bearing MVs. (**Paper III**)

3 ETHICAL CONSIDERATIONS

3.1 PAPER I

The experiments for paper I were conducted at the Institute of the Experimental Surgery and Biotechnology Research, Wroclaw University of Medicine. Ethical approval was obtained by the Animal Research Ethics Committee of the Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wroclaw, Poland.

When conducting animal experiments it is important to consider the tree R:s: Replacement, Refinement and Reduction.

Replacement means that animal experiments cannot be replaced by any other methodology. This is the case when studying a new combination of drugs. Even if both iNO and steroid are already registered drugs for humans in Sweden, it is important to see if this new combination does improve outcome and that it does not have any adverse effects. Also, steroid is a controversial drug in the context of sepsis. Pigs and humans have a similar physiology and therefore, pig is a reasonable animal when testing new drugs, before proceeding to humans. Although it is regrettable that animals die, it might be for the sake of saving even more human lives in the long run.

Refinement refers to that the animals should be well taken cared of before and during the procedure, so that they do not suffer. The subjects in study I were delivered from a farm and were hold during one night only at the veterinary hospital, where they were kept in a spacious facility and had free access to water. In order to avoid any possible suffering from the interventions during the study, animals were anesthetized. At endpoint, all animals were sacrificed in order to avoid any potential suffering caused by study interventions

Reduction denotes the need to keep down the number of animals for an experiment, in order to prove a scientific outcome. In our study power was calculated in accordance to a previous study [83] indicating that 6 animals per group would be enough. Although, the spread of the data was vaster than anticipated in study I and we could not prove any significances in the end, despite previous power calculations.

3.2 PAPER II-III

The study rendering paper II-III, were conducted at the Department of Clinical Sciences, Danderyd Hospital and were approved by the regional ethical review board at Karolinska Institutet, in Stockholm, Sweden.

It is however, reasonable to discuss the ethical considerations in administrating endotoxin to human volunteers. The short discomfort of the volunteers stands against that the endotoxemia model provides important information and insights regarding inflammatory conditions. Sepsis

is a major healthcare issue worldwide and could be hard to examine during the natural progression of the disease, as well as in cell lines or in animal models.

In a safety perspective, human models of endotoxin inflammation have been used for several years worldwide and our research group alone has experience from over 200 volunteers exposed to LPS. Neither in the literature have any significant or permanent adverse effects to the administration of LPS in such experimental set-ups, been reported. Nevertheless, the volunteers have been reported to sometimes experience vagal reflexes that can be easily lifted by adequate medical care. Therefore, a specialist in anesthesia and intensive care surveyed the subjects, all through the study time. Also, as an extra safety measure, the study was conducted in close proximity to the intensive care unit at Danderyd hospital and all volunteers did a health exam before being included in the study. For female participants the health exam was completed with a pregnancy test on the trial day.

Additionally, it is worth pointing out that all participants signed a written informed consent after a complete explanation of the study and *no* subject dropped out of this crossover trial. Moreover, in the end of the study, the volunteers answered the question anonymously if they would consider being included in a similar LPS study. *All* subjects said they would like to and for less of remuneration than they got for participating in this study.

3.3 ETHICAL CONCLUSIONS FROM A PERSONAL POINT OF VIEW

I think animal experiments are justified provided that

1. There is an ethical approval justifying the experiment.
2. The 3Rs are applied.
3. The results contribute to save even more human lives.

I find it acceptable to use endotoxin on human volunteers for the benefit of important knowledge, provided that the following criteria are met:

1. Ethical approval assuring that the trial is conducted according to Good Clinical Practice (GCP), including e.g adequate information to the volunteers and informed concent.
2. A health screening is conducted before inclusion, in order to assure healthy subjects.
3. Adequate monitoring of the volunteers by authorized personnel.

4 SUBJECTS AND METHODS

Table 2 summarizes the study designs from the papers included in this thesis.

Paper	Design	Subjects	Model	Randomization (N)	Outcome	Time Hrs	N =
I	RCT	Pigs	Endotoxin (LPS)	Control (6) LPS-only (6) LPS + iNO (6) LPS + IV steroid (6) LPS+iNO+IV steroid (6)	Organ function	30	30
II	Method validation	Volunteers	ELISA (LPS)	LPS/Placebo	H3Cit		22
III	Cross over	Volunteers	Endotoxin (LPS)	LPS/Placebo	H3Cit	7	22

Table 2: Study designs within the thesis

RCT = Randomized Controlled Trial, LPS = Lipopolysaccharide, iNO = inhaled Nitric Oxide, H3Cit = Citrullinated histone H3

4.1 SUBJECTS

4.1.1 Animals

In paper I, 30 domestic piglets, two months old and with a median body weight of 21 kg (range 15-24 kg) were studied. The pigs were fasted over night prior to the study.

4.1.2 Human volunteers

For paper II-III, healthy volunteers were recruited through advertisements posted in university campuses. Before inclusion, all volunteers got a health examination. A flow chart of the inclusion process is presented in figure 7.

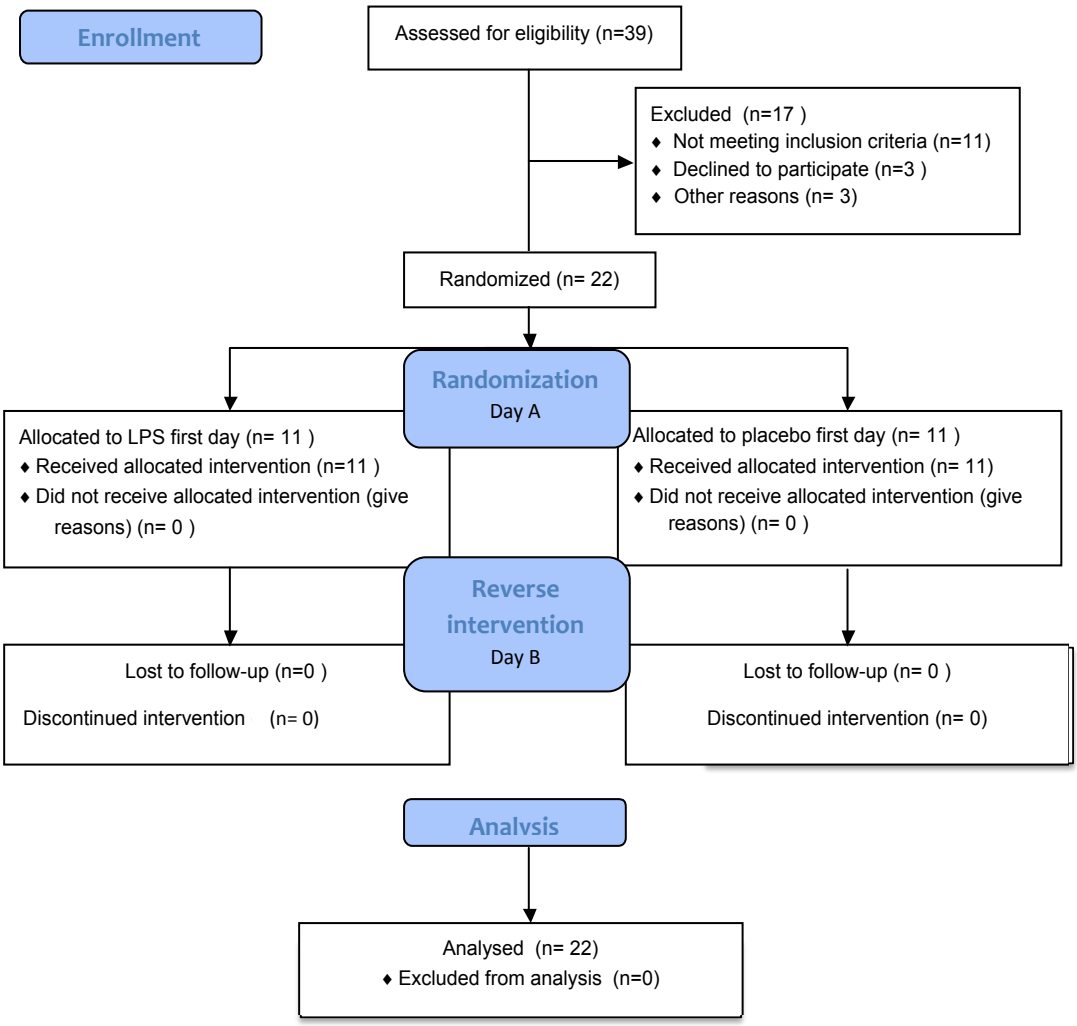

Figure 7. Flowchart illustrating the inclusion protocol for volunteers in paper II-III

Twenty-two volunteers were included in the protocol. Demographics are shown in the table 3 below:

Variable	Value
Age, Mean (range) years	23.4 (19-34)
Male No (%)	13 (59%)
Weight mean (range) kg	70 ± (50-104)
BMI, mean (range) kg/m^2	22.9 ± 18-32
Smokers No (%)	0 ± 0
Drop outs No (%)	0 (0)

Table 3. Demographics of healthy volunteers included in paper II and III

4.2 ENDOTOXIN (LPS) ADMINISTRATION

4.2.1 Paper I

A septic shock-like condition was established by *continuous intravenous infusion* of endotoxin (Lipopolysaccharide (LPS) from E. coli (L2630-25MG, SIGMA, Gothenburg, Sweden, Chemical lot 110K4110, mixed in sterile water = 2 mg/ml). An initial dose of 5 μg/kg/hr was administered for two hours after baseline measurements and then lowered to 1μg/kg/hr, for the remaining 28 hours of the study period. An infusion allows for maintenance of the endotoxin shock during the 30 hr of study time.

4.2.2 Paper II-III

An *intravenous injection* of LPS 2 ng/kg endotoxin from E. Coli (Lot H0K354 CAT number 1235503, United States Pharmacopeia, Rockville, MD, USA) was used, producing transient flue-like symptoms (headache, nausea, muscle pain, fever) lasting for about 7 hrs. The LPS powder was dissolved in 0.9 % physiological saline (NaCl) and treated for 10 min by ultrasound (Bransonic 3510, Bransonic Ultrasonic Corp, Danbury, USA) before administration in order to avoid flocculation.

4.3 RANDOMIZATION AND STUDY DESIGN

The randomization for both studies was performed using sealed envelopes.

For **study I** a randomized controlled study design (RCT) with parallel groups was used. The 30 piglets were randomized into 5 groups and observed for 30 hrs as follows:

1. **Control**: Anesthesia and mechanical ventilation without LPS infusion, iNO, or IV steroid. (N = 6)
2. **LPS-only**: Continuous IV LPS infusion for the 30 hr study period. (N = 6)
3. **LPS + iNO**: iNO at 30 ppm started after 3 hrs of IV LPS infusion and continued until the end of the experiment. (N = 6)
4. **LPS + IV steroid**: IV hydrocortisone 75 mg started 3 hrs after IV LPS infusion and repeated every 8 hrs thereafter. (N = 6)
5. **LPS + iNO + IV steroid**: Both continuous iNO at 30 ppm and IV hydrocortisone 75 mg every 8 hrs starting after the initial 3 hrs of IV LPS infusion. (N = 6)

A timeline illustrating study I, is presented in figure 8.

For the human endotoxin study, rendering **paper II-III,** a double-blinded, randomized placebo-controlled, within-subject (cross over) design was used (fig 9). The 22 healthy human volunteers were randomly assigned to receive either a **LPS injection or** the same volume of **0.9 % NaCl** on the first session of the study. Subjects were studied for 7 hrs.

After 3-4 weeks of wash out period, the second session occurred in which subjects were assigned the reverse treatment.

4.4 EXPERIMENTAL PROTOCOLS

4.4.1 Specific protocol paper I

The protocol was designed to evaluate the long-term effects of LPS on organ function, by various interventions.

A flowchart describing the various experimental procedures is given in figure 8. Fluid and vasopressor supports were administered as needed (see below) to all groups.

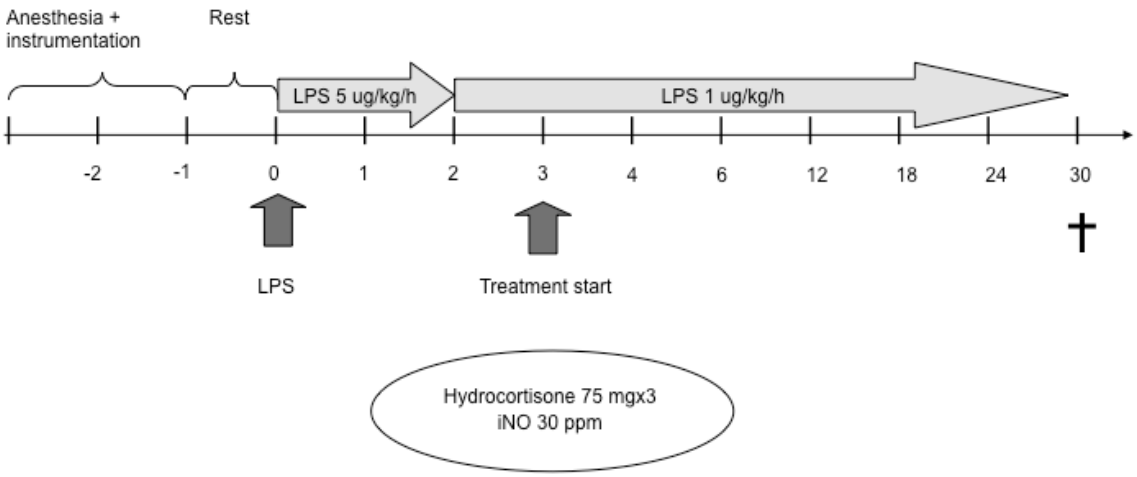

Figure 8. Experimental protocol paper I. *Reprinted from Göranson SP, Goździk W, Harbut P, Ryniak S, Zielinski S, Haegerstrand CG, et al. (2014) Organ Dysfunction among Piglets Treated with Inhaled Nitric Oxide and Intravenous Hydrocortisone during Prolonged Endotoxin Infusion. PLoS ONE 9(5): e96594. https://doi.org/10.1371/journal.pone.0096594. © 2014 Göranson et al. With permission under the terms of the Creative Commons Attribution License, open access.*

An LPS infusion of 5 µ/kg/hr was started at time 0 after instrumentation on anesthetized animals. Infusion was lowered to 1 µg/kg/h after 2 hrs and was kept on this level until the end of the study time at 30 hrs, when all animals were sacrificed. Treatment with either iNO, IV steroid (hydrocortisone) or a combination of both, was initiated 3 hrs post LPS infusion start.

LPS = Lipopolysaccharide; iNO = inhaled nitric oxide: IV = intravenous; ppm = parts per million; µg = microgram; kg = kilogram

4.4.1.1 Anesthesia and instrumentation

Anesthesia induction was performed by an intramuscular injection of zolazepam/tiletamin 4 mg/kg dissolved in medetomidine 0.08 mg/kg. Piglets were intubated and subsequently ventilated using a pressure-controlled mode on a Servo 900C ventilator (Siemens Elema-Solna, Sweden). Ventilator was set at an inspired fraction of oxygen (FIO_2) of 0.3, a PEEP of 5 cm H_2O and the inspiratory pressure aimed to keep the piglets normo-ventilated.

For maintenance of the anesthesia an IV infusion of a mixture of ketamine 1.5-2.4 mg/kg/hr, medetomidine (5.3-8.2 µg/kg/hr), fentanyl (0.8-1.3 µg/kg/hr), and midazolam (0.08-0.13 µg/kg/hr) was used. During instrumentation, anesthetic doses were increased in order to avoid stress. Anesthesia doses were then lowered to standard sedation doses, for the rest of the study time. Instrumentation included insertion of a femoral arterial line (BD Careflow

femoral artery catheter®, Becton Dickinson, Singapore, Singapore) and a supra-pubic urinary bladder catheterization by a mini laparotomy (Rüsch catheter, Kernen, Germany). By internal jugular cut down both a balloon tipped flotation catheter with a thermistor (PAC) (CritiCath SP5105H TD catheter®, Becton Dickinson, Singapore, Singapore) as well as a central venous catheter (CVC) (BD Careflow central venous catheter®, Becton Dickinson, Singapore, Singapore) were inserted. The tip of the PAC was advanced into the pulmonary artery. In order to evacuate any ascites fluid, a drainage tube was finally inserted into the abdominal cavity. Baseline data (=time zero) were registered after a 1hr recovery period and the blood was sampled from the arterial catheter.

4.4.1.2 Administration of iNO and IV steroid

Inhaled nitric oxide (iNO) (Pulmonox-Messer Griesheim 800 ppm NO in 9000 nitrogen) was delivered at 30 ppm by a Pulmomix Mini (Messer Griesheim, Gumpoldskirchen, Austria) to the inspiratory limb of the ventilator as described previously [101].

4.4.2 Specific protocol paper II-III

The protocol was designed to assess the effect of LPS on circulating H3Cit.

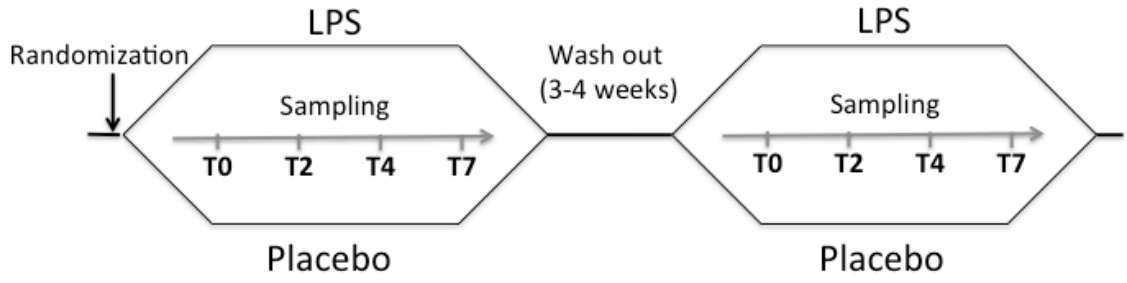

Figure 9. Experimental protocol and study design paper II-III

After randomization subjects received either an LPS or a saline injection intravenously. Plasma analyzed was sampled at baseline (T0) and 2, 4 and 7 hrs post LPS injection. After 3-4 weeks of washout, volunteers were subjected to the inverse intervention.

LPS= Lipopolysaccharide (2 ng/kg), Placebo = Saline (NaCl) 0,9%, T= Time in hrs after injection, Sampling= Blood sampling for microvesicles, citrullinated histone H3, white blood cell count and cells, cytokines and full blood count.

After arrival the subjects had two intravenous catheters. One was used for the LPS/saline injection (22G) and was removed immediately after the injection. The other was placed in the opposite antecubital fossae (17G) and was used for blood sampling. All volunteers and research staff were blinded, except the physician involved in the study for security purposes.

4.5 MONITORING

4.5.1 Monitoring paper I

Continuous monitoring included pulse oximetry (SpO_2), a three-point electrocardiogram (ECG), recordings of heart rate (HR), end-tidal carbon dioxide concentration ($EtCO_2$) (General Electric health care AS/3 Instrumentarium, OY Helsinki, Finland), fraction of inspired oxygen (FIO_2). pulmonary arterial-, central venous- and mean systemic pressures, (MPAP, CVP and MAP). Thermo-dilution cardiac output (CO, L/min) and pulmonary capillary wedge pressure (PCWP, mmHg) were measured every 4 hrs. Pulmonary- (PVRI) and systemic (SVRI) vascular resistance indexes (dynes-sec /cm^{-5}/m^2) were calculated out of the measurements obtained from the PAC and artery line. Urinary output was registrated and the urinary reservoir emptied after 12, 24, and 30 hrs of endotoxin infusion. The PAC thermistor recorded central body temperature, in order to keep the animals normothermic (37-38°C). Hypothermia was avoided by use of heating blankets, or external cooling when necessary. Every 4 hrs, the animals were turned side to side.

MAP less than 60 mmHg for longer than 3 min was defined as hypotension in need of treatment. Hypotension treatment started with a lactated Ringers solution bolus, or a rapid infusion of hydroxyl ethyl starch (HES). HES was given at a maximum dose of 750 ml. Norepinephrine infusion (40 μg/ml) was initiated if fluid bolus was inefficient in restoring MAP to > 60 mmHg. Central venous pressure (CVP) aimed to be between 6 and 8 mmHg.

In order to maintain the blood glucose and to compensate for fluid loss, a mixture of 2.5% glucose in saline 0.9% (Glucose/NaCl 1:1, Braun, Melsungen, Germany) at a basal infusion rate of 100 ml/hr, was administered during the study time. Low or high blood glucose levels were not treated. In order to counteract bradycardia, Glycopyrrolate 0.2 mg was mixed into the infusate of 1500 mL. Additionally, cefuroxime (GlaxoSmithKline, Solna, Sweden*)* was administered at a dose of 500 mg IV every 8 hrs, in order to avoid septicemia due to accidental bacterial contamination from instrumentation.

4.5.2 Monitoring paper II-III

Visual Analog Scale (VAS) was used for assessment of nausea, headache and lower back pain every 30 min throughout the experiment. Tympanic temperature (ThermoScan pro 1, Thermoscan Inc. San Diego, USA), respiratory rate, peripheral oxygen saturation (SpO2), pulse, ECG monitoring, non- invasive blood pressure (Philips Intellivue X2, Boeblingen Germany) were registered.

4.6 SAMPLING

4.6.1 Specific blood sampling paper I

After instrumentation and a subsequent recovery for one hour, blood samples were drawn, from the artery line, at baseline, 0 hr (immediately prior to starting LPS infusion and then at 6, 12, 24, and 30 hrs after baseline. Samples included white blood cell cell (WBC) count, interleukin 1 and 10 (IL-1, IL-10), tumor necrosis factor α (TNF-$_{\alpha}$), alanine aminotransferase (ALAT), creatinine, urea, sodium (Na), potassium (K), chloride (Cl), and platelet count. Point-of-care analysis of blood gases (ISTAT, Abbot, East Windsor, USA) included pH, $PaCO_2$, PaO_2, bicarbonate (HCO_3^-), lactate, and base excess (BE). High or low potassium levels and acidosis were not treated. Blood drawn for interleukins was immediately centrifuged (Hettich Zentrifugen Universal 16R, Hettich Gmb&H, Tuttlingen, Germany) for ten min at 4000 g/min. The supernatants were stored at -70ºC until analyzed. The remaining parameters (WBC, ALAT, creatinine, urea, Na, K, Cl, and platelets) were analyzed within one hr.

4.6.2 Specific blood sampling paper II-III

Blood samples were taken through an intravenous cannula at baseline (0 hr) and then at 1 hr, 1.5, 2, 3, 4, 5 and 7 hrs post baseline. If blood could not be drawn easily from the cannula, direct blood sampling was performed. However, papers presented in this thesis only include analyzed blood samples from 0, 2, 4 and 7 hrs post LPS injection.

4.6.2.1 Microvesicles and citrullinated histone H3

Samples from sodium citrate tubes were centrifuged immediately at 2000g for 20 min at room temperature and the samples were stored as platelet-poor plasma at -80°C until analysis for MVs and H3Cit.

4.6.2.2 Cytokines, blood count and cells

Blood samples from EDTA tubes were centrifuged and plasma was aliquoted and stored at -80°C until the analyses. Plasma levels of the inflammatory cytokines, IL-6, TNF-α and IL-8, were measured using multiplex assays (Human Mag Luminex Performance Assay, LHSCM000, LHSCN206, LHSCM208, LHSCM210, RnD Systems, MN, USA).

4.7 SPECIFIC METHODS

4.7.1 ELISA (paper II-III)

Due to the lack of objective and quantitative methods to assess levels of citrullinated histone H3 (H3Cit) as a marker for systemic NET burden, a novel ELISA-based assay (paper II) was methodologically validated.

In lack of samples of known concentrations, we chose to use plasma samples obtained from the human endotoxin study described above, assuming that inflammation would induce a systemic NET formation resulting in elevated levels of H3Cit in plasma. This hypothesis was based on previous results by others, demonstrating detectable H3Cit levels by western blot in a mouse model, 3 hrs post LPS injection [64]. Consequently, in line with these data, human plasma samples 3-4 hr post IV injection of LPS (2 ng/kg of body weight) assumed to be suitable for the H3Cit assay validation.

An anti-histone antibody served as a capture antibody for the assay. For detection an anti-histone H3 citrulline antibody was used (fig 10). A standard curve was obtained, using in vitro PAD4-citrullinated H3Cit [113]. Incubation times, dilutions of samples and the concentrations of the standard curve were optimized in preliminary experiments.

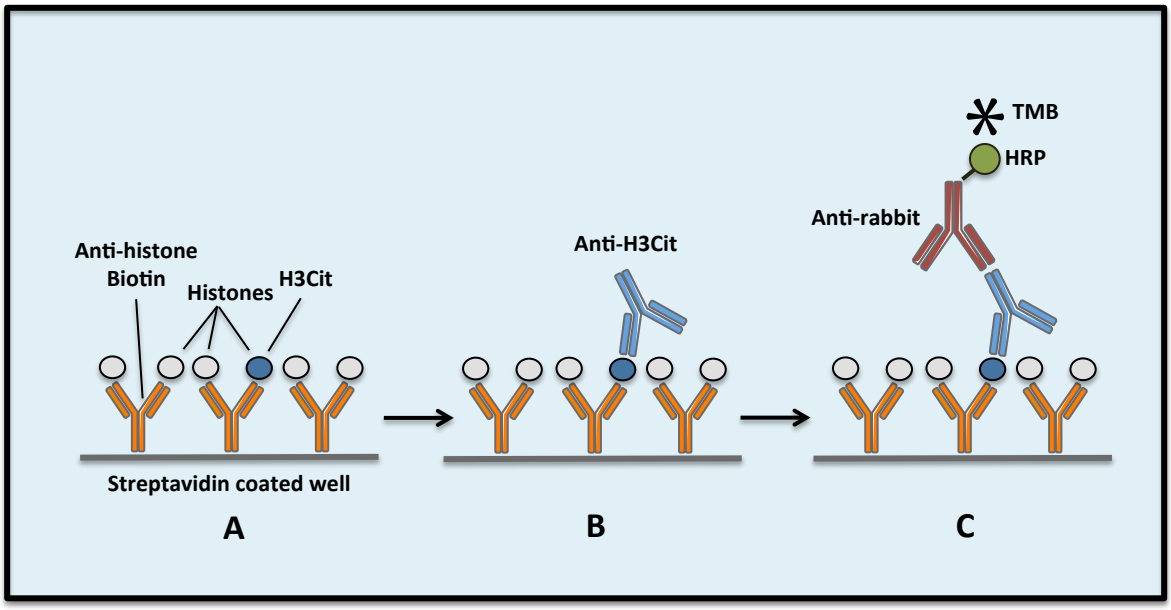

Figure 10. Schematic of the H3Cit ELISA. *Reprinted from Thålin, C., Daleskog, M., Göransson, S.P. et al. Immunol Res (2017) 65: 706. https://doi-org.proxy.kib.ki.se/10.1007/s12026-017-8905-3. © Thålin et al 2017, with permission under the terms of the Creative Commons Attribution License, open access.*

A – To a streptavidin coated well, the anti-histone Biotin (capture antibody) is added and incubated. Plasma samples are pipetted into the wells. Histones present in the sample bind to the capture antibody during a second incubation. **B -** After washing, an anti-H3Cit antibody is added to the wells. In a third incubation, Anti-H3Cit binds to citrullinated histone H3 (H3Cit) but not to non-citrullinated histone H3. **C -** A horseradish peroxidase (HRP) conjugated anti-rabbit antibody is added in a fourth incubation. This antibody binds to the Anti-H3Cit. Finally, Tetramethylbenzidine (TMB) is added for detection.

H3Cit = Citrullinated histone H3; HRP = Horse Radish Peroxidase; TMB = Tetramethylbenzidine

The assay was evaluated in accordance with recommended ELISA validation requirements [114] for the following:

1. *stability*,
1. *linearity*
2. *limit of detection,*
3. *specificity,*
4. *recovery/effect of the matrix,*
5. *precision and reproducibility.*

Trueness could not be determined as there is no available assay or reference analyte of known concentration for comparison.

Stability was assessed by comparing the standard curves for three different batches of frozen H3Cit standard, prepared on three different days. A freshly prepared H3Cit standard was also compared with a frozen aliquot.

The suitable **linear interval**, i.e. the measuring interval, was defined as the linear section of the best-fit standard curve. It was determined by interpolating the detected optical density (O.D.) from serial dilutions of H3Cit to different regressions. The 95% confidence interval (95% CI) was considered.

The **limit of detection** was first approximated by assessing the minimum level at which H3Cit could be detected in serial dilutions of positive samples. Each standard curve was fitted using a four-parameter logistic (4PL) regression to the calibration curve and the 95% CI was considered. The limit of detection was finally set by comparing the approximated level to the concentration corresponding to the highest O.D. at the bottom 95% CI.

Specificity was assessed by the ability to detect citrullinated histone H3 in the presence of non-citrullinated histone H3. A standard without PAD4 was prepared, thus preventing the citrullination of histone H3.

Recovery and the **effect of the matrix** were assessed by spiking plasma samples from four healthy volunteers with known concentrations (625, 312, 156, 78, 38, 19 and 10 ng/ml) of in vitro PAD4-citrullinated H3Cit.

To further assess the effect of the matrix, the standard was prepared in healthy plasma diluted in Phosphate-Buffered Saline (PBS); dilutions 1:20, 1:10 and 1:5.

Precision/Reproducibility was assessed by measuring six replicates of eight samples within the same assay (intra assay) and by comparing the same eight samples (in duplicates) in four different assays runs, on four different days (inter assay). Reproducibility was considered acceptable if the coefficient of variation (% CV), defined as the ratio between standard deviation and mean value) was less than 15%.

4.7.1.1 Detection of plasma H3cit by ELISA (Paper III)

Plasma samples were thawed on ice. H3Cit was quantified by the capture ELISA described and validated in paper II and explained in detail above. Briefly, H3Cit levels were obtained

by use of an anti-histone antibody as capture antibody (Roche, Basel, Switzerland), and subsequently an anti-histone H3 citrulline antibody for detection (Abcam, Cambridge, UK). To measure the portion of MV-bound H3Cit in the samples, the same ELISA assay analysed H3Cit in an MV-free supernatant and an MV-enriched pellet after high-speed centrifugation of the plasma.

4.7.2 Flow cytometry (paper III)

Flow cytometry is the golden standard for measuring MVs, due to its ability of measuring multiple protein expression. This ability allows for phenotyping (speciate) the MV.

A schematic figure of the flow cytometer is shown in figure 11

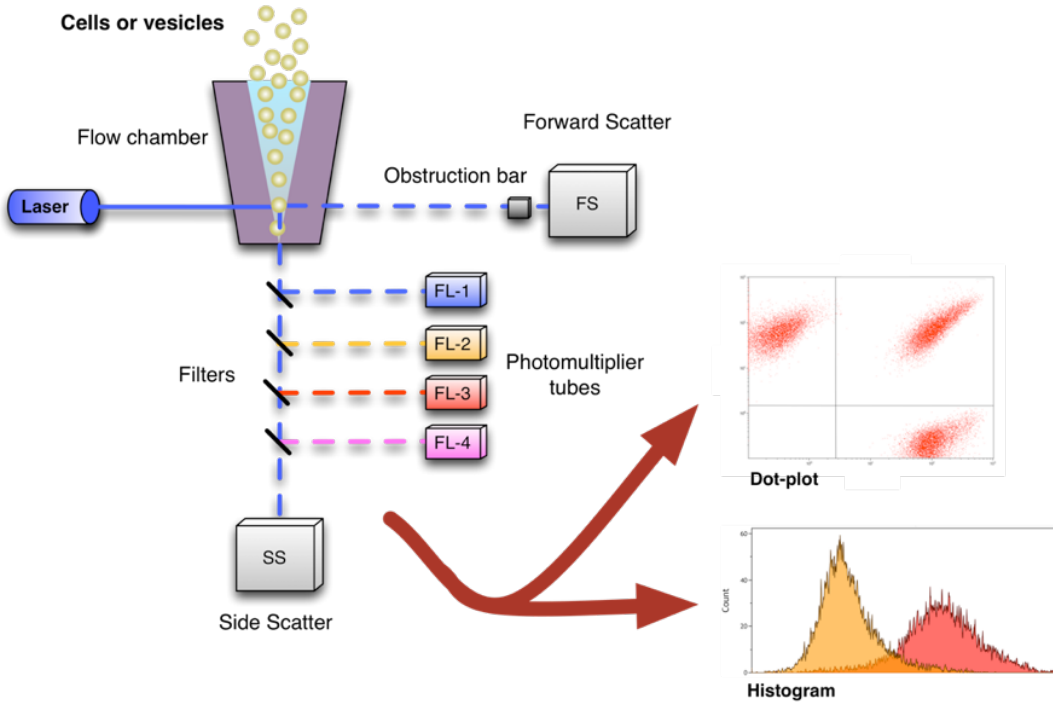

Figure 11. Basic principals of flow cytometry. *Reproduced with permission from Fariborz Mobarrez, PhD.*

The MVs pass through a flow chamber filled with PBS. A laser beam spots the MVs as the flow chamber narrows down, producing both a forward scatter (FSC),"measuring size", and a side scatter. The nature of the scattered laser depends of the size and complexity of the particle.

If an MV is conjugated with a fluorescent antibody, it can be detected by the flow cytometer by its fluorescent signal emissions, creating different colors of the light beams. This allows for the MV to be phenotyped. Several cell properties can further be measured simultaneously by labeling MVs with multiple antibodies, each with a different fluorochrome or "color". [115]. Examples of antibodies used within this thesis are CD66b and myeloperoxidase

(MPO) for neutrophil detection and CD42a for platelet detection, as well as anti-H3Cit antibody for detection of H3Cit-bearing MVs. Fluorochromes used were allophycocyanin (APC), phycoerythrin (PE) and fluorescein isothiocyanate (FITC).

The light emissions are detected and digitized for analysis by a computer and are usually displayed in dot-plots or histograms.

4.7.2.1 Detection of plasma MVs by flow cytometry

Plasma samples were centrifuged at 2000 g for 20 min at room temperature (RT), after being thawed once in water bath, 37° C. Re-centrifugation of the supernatant was performed at 13000 g for 2 min at RT. Thereafter, 20 µl of the supernatant was incubated for 20 min in dark, with 5 µl of anti-histone H3 citrulline antibody (Abcam, Cambridge, UK) in order to detect H3Cit-bearing MVs, CD66b APC (Beckman coulter, Brea, CA, USA) and MPO PE for the detection of neutrophil-derived MVs, as well as CD42a FITC (Beckman coulter, Brea, CA, USA) for the detection of platelet-derived MVs (Beckman coulter, Brea, CA, USA). Levels of MVs were detected by flow cytometry on a Beckman Gallios instrument (Beckman coulter, Brea, CA, USA). The use of Megamix-Plus FSC beads (0.3, 0.5 and 0.9 µm in size; BioCytex, Marseille, France) was used in order to determine the MV gate. MVs were defined as particles less than 0.9 µm in diameter (forward scatter). As negative controls, conjugate isotype matched immunoglobulins, with no reactivity against human antigens were applied. In this thesis, results are shown as numbers of MVs (MV counted x standard beads added/L)/standard beads counted (FlowCount, Beckman Coulter, CA, USA). The inter- and intraassay coefficients of variation for MV measurement were less than 9% respectively.

4.7.2.2 Incubation of purified H3Cit with in vitro generated platelet-derived MVs

The binding properties of H3Cit to MVs were examined by experiments in vitro. From fresh platelets extracted from healthy donor blood, a high concentration of platelet-derived MVs was generated by centrifugation. In short, in order to obtain a platelet-rich plasma (PRP), citrated whole blood was centrifuged at 200 g for 10 min at RT. The platelet agonist ADP (35 µM, Roche, Basel, Switzerland) was then used to activate the PRP, during 20 min at RT, rendering platelet-derived MVs. When activated, the PRP was centrifuged at 2000 g for 20 min at RT in order to separate platelets from the supernatant with platelet-derived MVs. The supernatant was then re-centrifuged at 20 800 g for 45 min at RT twice (phosphate-buffered saline wash in between). By removing the supernatant, an MV-enriched pellet was created and was aliquoted into three samples (N = 3). The MV-enriched samples were labeled with lactadherin and anti-histone H3 citrulline, thereby determining baseline levels of PS exposure and H3Cit. Briefly, each sample measuring 20 µl, was labeled with 5 µl of lactadherin FITC (Haematologic Technologies, VT, USA) and anti-histone H3 citrulline Dylight 755. The samples were then measured by flow cytometry (described above), after 20 min incubation in RT. The MV-enriched samples were subsequently incubated with increasing concentrations of H3Cit peptide (abcam, Cambridge, UK), 0 – 1500 ng/ml, for 90 min at 37 °C. The samples were once again labeled with lactadherin FITC as well as with anti-histone H3 citrulline

Dylight 755 and measured by flow cytometry. MVs were phenotyped based on their PS exposure and expression of H3Cit and were defined as particles less than 0.9 μm in size (forward scatter).

5 STATISTICAL ANALYSES

Appropriate statistical method was chosen according to the nature of the data and was adjusted to fit the study design. Normally distributed data was presented as mean and standard deviation (SD). Skewed data were presented as median and range/interquartile range (IQR). A p-value of < 0.05 was considered significant.

5.1 SPECIFIC STATISTICS PAPER I

The main research question in paper I was to identify differences between the different treatment groups (N = 3) vs LPS-only in a randomized control study design (independent data).

Due to the low sample size, in combination with mostly skewed data, non-parametric tests were used. To evaluate the treatment effect over time, a Wilcoxon signed rank test was used to compare the difference between end-point (30 hrs) vs baseline for each group. A Wilcoxon-Mann-Whitney test was then performed in order to compare the difference in treatment effect between groups at endpoint. Due to multiple group comparisons (LPS vs 1. LPS + iNO; 2. LPS + steroid and 3. LPS + iNO + IV steroid) a Bonferroni Holm correction was subsequently performed.

Further, a Kruskal Wallis was performed for all base line data, in order to assure a non-significant difference between the groups before randomization.

For subjects that died, but lived until at least 20 hrs post start of LPS infusion, last-observation-carried-forward method, based on the intention-to-treat principle was applied. This was the case for 2 out of 3 deaths.

5.2 SPECIFIC STATISTICS PAPER II

The aim of paper II was to validate an H3Cit ELISA.

The nominal log concentration was fitted versus O.D., applying a sigmoidal 4PL regression to the calibration curve in order to specify linearity. 4PL curves were then compared by F-test.

In order to demonstrate precision and reproducibility, the variation of inter- and intra-assay experiments were presented as coefficient of variation (CV). CV was defined as the ratio of the SD to the mean (%).

5.3 SPECIFIC STATISTICS PAPER III

The aim of paper III was primarily to study dynamics of the biomarker H3Cit over time in a cross over design (dependent data).

In order to adjust for multiple time points a repeated measures ANOVA was used if the data was normally distributed. If data were skewed, despite log transformation, a generalized mixed model (GEE) was used. The ANOVA or GEE was based on the differences between LPS- placebo for each time point (dependent data). The covariate ”trial day” (the day the subject got LPS) was applied in order to prove sufficient wash out from LPS. Further, the covariate ”time” (change over time) was included in the repeated measures model.

Post hoc comparison was allowed for if the repeated measures model was significant. A Wilcoxon signed rank test was used both for normally and not normally distributed data, due to the relatively low number of subjects in the study (N = 22) in line with the central limit theorem. Since the dynamics over time were the main focus of interest, statistics were analyzed mainly for the change over time between the LPS versus the placebo arm.

6 RESULTS

This section will summarize the results in brief and as an overview. Detailed information is presented in the separate papers. Results presented in this part will focus on organ function and H3Cit/MV response.

6.1 COMPARISON OF REACTIONS TO ENDOTOXIN ADMINISTERED AS INFUSION OR INJECTION

6.1.1 General physiological symptoms from endotoxin

When IV LPS was given in a μg dose and as an infusion to piglets (**paper I**) more pronounced and sustained physiological changes were apparent compared to the administration of a single injection IV in a ng-dose to humans (**paper II-III**). Exposure to the LPS infusion caused a decrease in MAP, increased peak inspiratory pressure, impaired gas exchange and elevated serum creatinine in pigs. Meanwhile, healthy human volunteers injected with a single ng-dose of LPS IV, experienced headache, nausea, chills and fever. The symptoms started promptly at 1 hr post LPS injection and were most intense during the subsequent hr. After 7 hrs symptoms had more or less abated. No significant difference in hemodynamics was seen between the LPS and the placebo arm, except for heart rate increasing significantly with temperature at 3 hrs.

6.1.2 White blood cell count and platelets

In accordance with previous data [94] a drop in white blood cell (WBC) count were seen in humans after LPS IV injection, in which the decrease corresponded to a drop in neutrophil count (fig 12D). Also pigs with a continuous IV infusion of LPS exhibited a drop in WBC count, but later, after 6 hrs of LPS infusion (12A), as shown in figure 12. Interestingly groups treated with iNO (LPS + iNO and LPS + iNO + IV steroid) did not exhibit this drop.

Platelets decreased significantly over time ($p < 0.001$) in LPS treated groups in both LPS injected humans (fig 12E) and pigs exposed to LPS infusion. Interestingly, also the healthy control animals experienced a drop in platelets (fig 12B). Leukocytosis was more pronounced in pigs after the higher dose of LPS as infusion (fig 12A) than after the IV injection of 2ng/kg given to humans (fig 12C).

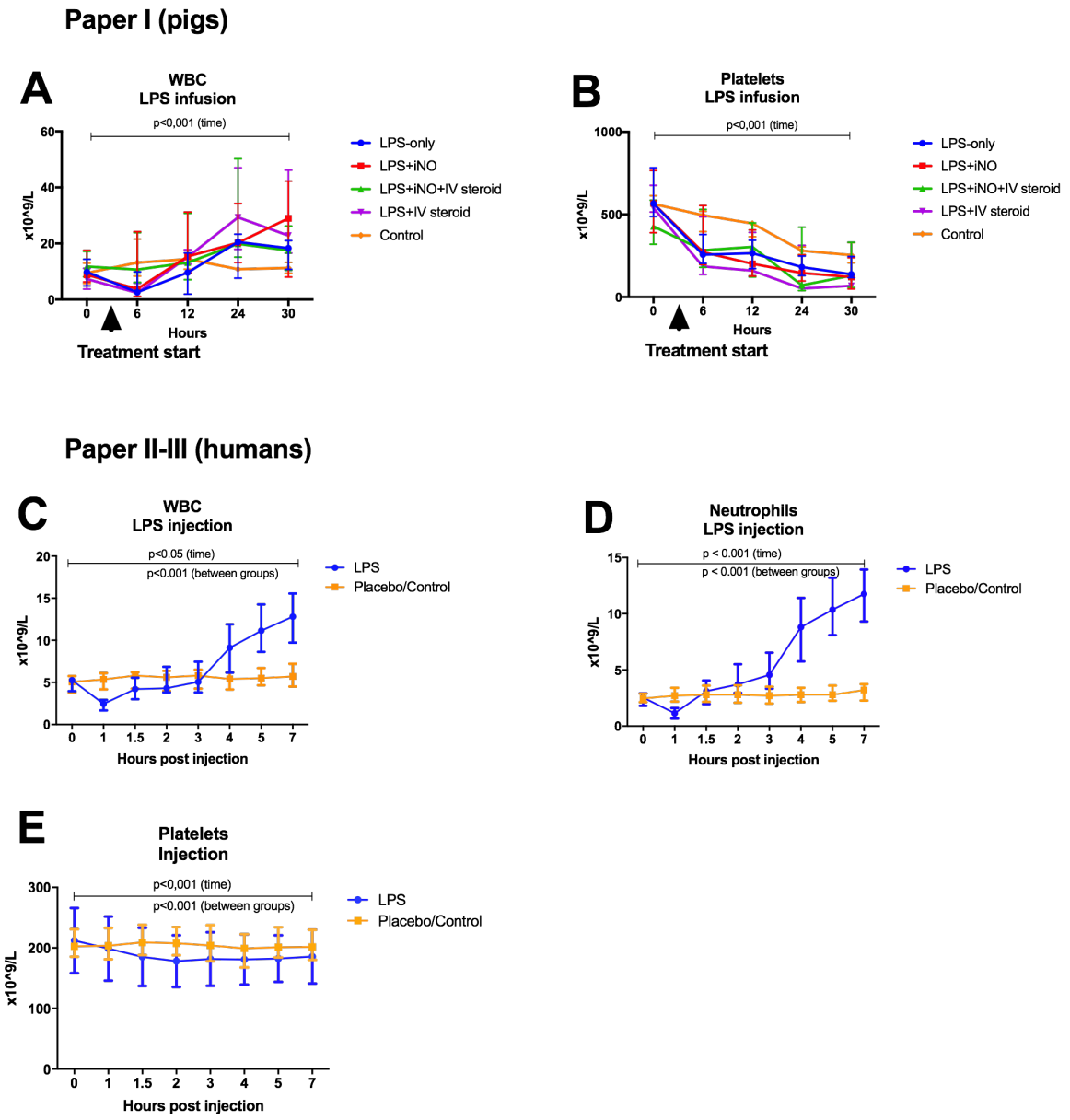

Figure 12. White blood cell count and platelets in LPS infusion (pigs) (A-B) compared to LPS injection (humans) (C-E).

There was a drop in white blood cell count with a subsequent leukocytosis after endotoxin infusion (A) as well as after injection (C). This drop corresponded to a drop in neutrophils as illustrated for the human data (D). Platelet levels decreased significantly over time ($p < 0.001$) for LPS infusion (B) and LPS injection (E).

WBC = White blood cell count; LPS=Lipopolysaccharide; iNO = inhaled nitric oxide; IV = intravenous; Control = sham animals or humans exposed to saline injection, no LPS given; time = change in plasma-levels of WBC/neutrophils/platelets over the study time; Data are presented as median and intraquartile range (IQR); N = 22 for humans and 30 (6 /group) for animals.

6.1.3 Cytokine response

Further, cytokines were significantly elevated after endotoxin in LPS treated individuals. No significant dynamics were seen for any cytokine measured in the studies. All cytokines (TNF-α, IL-1β, IL-10 in paper I and TNF-α, IL-6 and IL-8 in paper II-III) were significantly elevated over time after endotoxin administration.

During continuous infusion in pigs IL-10 and TNF-α levels peaked at 6 hrs whereas IL-1β did not peak until 12 hrs. At the 30 hr endpoint, all cytokines approached baseline.

In humans TNF-α was the first cytokine to peak at 1.5 hrs after LPS injection of 2 ng/kg, corresponding to highest VAS score for headache followed by a subsequent simultaneous peak of IL-6 and IL-8 at 2-3 hrs post LPS injection that matched fever. All cytokines had returned to baseline at the 7 hr endpoint.

Differences in TNF-α levels when comparing infusion in pigs and injection in humans, are illustrated in figure 13 and were in accordance with previous reported data [87]. TNF-α profile changed during infusion of LPS compared to after LPS injection. During infusion TNF-α peaked later (at 6 hrs) compared to when administered as an injection when the peak was at 1.5 hrs. Higher dose of LPS generated a more pronounced response in TNF-α.

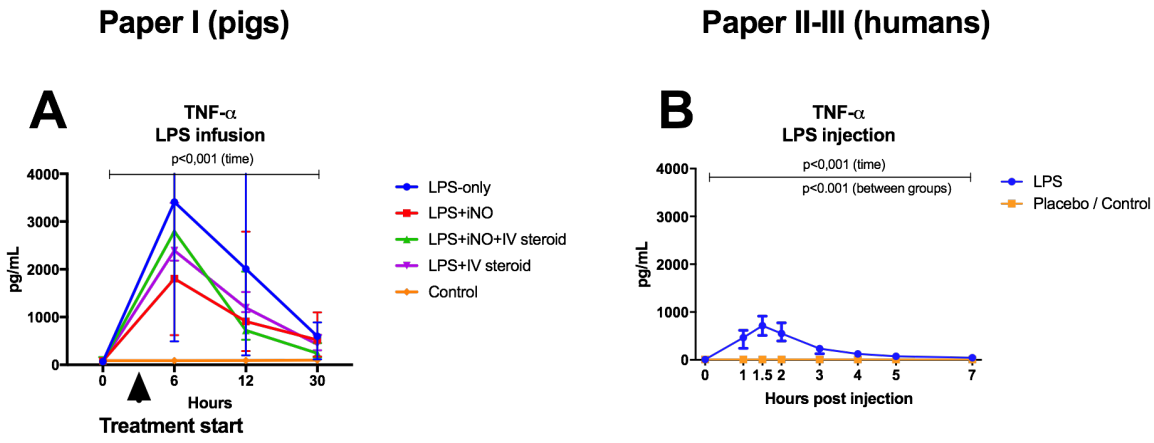

Figure 13. TNF-α levels in pigs during LPS infusion (5 µg/kg lowered to 1µg/kg) (A) compared to after LPS injection (2ng/kg) in humans (B)

A) During continuous LPS infusion in pigs, TNF-α peaked at 6 hrs and did not quite reach baseline in the end of the 30 hrs study time. There was a trend towards lower TNF-α levels in the treatment groups compared to LPS alone, but no significant difference was seen. **B)** TNF-α levels peaked earlier at 1.5 hrs after LPS injection of 2 ng/kg in humans than during infusion. Baseline levels of TNF-α were noted 7 hrs post LPS injection in humans. TNF-α = Tumor necrosis factor α; LPS = Lipopolysaccharide; iNO = inhaled nitric oxide; IV = intravenous; Control = sham animals or humans exposed to saline injection, no LPS given; pg/mL= pikogram/milliliter; time = change in TNF-α-levels over the study time; Data are presented as median and IQR. N = 22 for humans and 30 (6/group) for animals.

6.2 EFFECTS OF INO + IV STEROID IN A PORCINE ENDOTOXEMIA MODEL (PAPER I)

6.2.1 Mortality and causes

There was no death in the Control group or in the LPS + iNO + IV steroid group. Other LPS exposed groups had 1 case of death each. Details are presented in table 4.

	Control	LPS-only	LPS + IV steroid	LPS + iNO	LPS + iNO+IV steroid
Deaths N	0	1	1	1	0
Timepoint of death		6 hrs	20 hrs	18 hrs	
Cause of death		High MPAP with respiratory failure	Severe shock	Circulatory failure	

Table 4. Mortality details paper I

LPS = Lipopolysaccharide; iNO = inhaled nitric oxide; IV = intravenous; MPAP = mean pulmonary artery pressure; ; Control = sham animals, no LPS given; N = number of deaths out of 6 animals per group; hrs = hours

6.2.2 Renal function

Renal function after LPS infusion is summarized in figure 14 below:

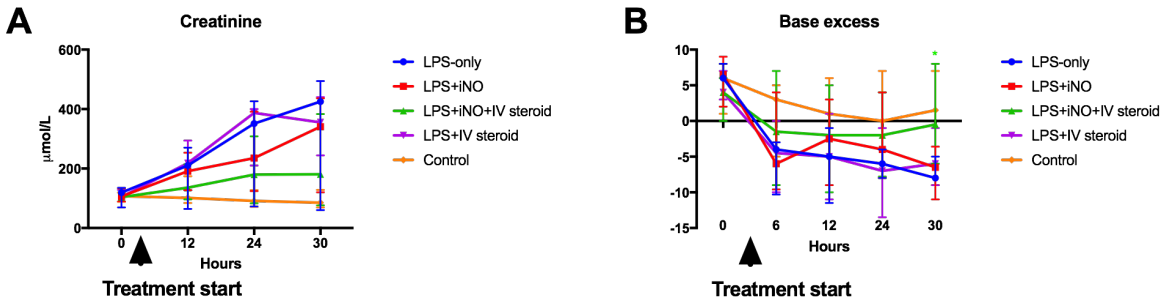

Figure 14. Renal failure after endotoxin infusion in pigs tended to be less in the LPS + iNO + IV steroid group when compared to LPS-only.

A) Creatinine tended to be lower in LPS + iNO + IV steroid treated pigs compared to LPS, but there was a big spread in the data. A significant different could be seen between LPS alone and control ($p < 0.05$). No significances were seen between other groups compared to LPS alone. However, B) Base excess was significantly higher/better in LPS + iNO + IV steroid treated pigs compared to LPS-only.

LPS = Lipopolysaccharide; iNO = inhaled nitric oxide IV = intravenous. Control = sham animals, no LPS given; Data are presented as median and range. N = 30 (6/group) * $p < 0.05$ = Comparison of distribution of differences versus LPS-only (Bonferroni-Holm adjusted p-value); Data are presented as median and intraquartile range; N = 30 (6/group).

6.2.3 Hemodynamics

In order to obtain a MAP of a minimum of 60 mmHg, initially fluids were infused to a CVP up to 6-8 mmHg. If MAP was still less than 60 mmHg, norepinephrine was titrated. There was no significant difference between groups in the amount of fluids administered. However, the total amount of norepinephrine given tended to be lower in the LPS + iNO + IV steroid group compared to the LPS-only group ($p < 0.05$; Bonferroni-Holm corrected $p = 0.1$) (fig.15). Further, the LPS + NO + IV steroid group only required norepinephrine during the initial 4 hrs of the study time (1 hr post treatment start). LPS + IV steroid showed a similar pattern, but 2 pigs required norepinephrine even after 4 hrs.

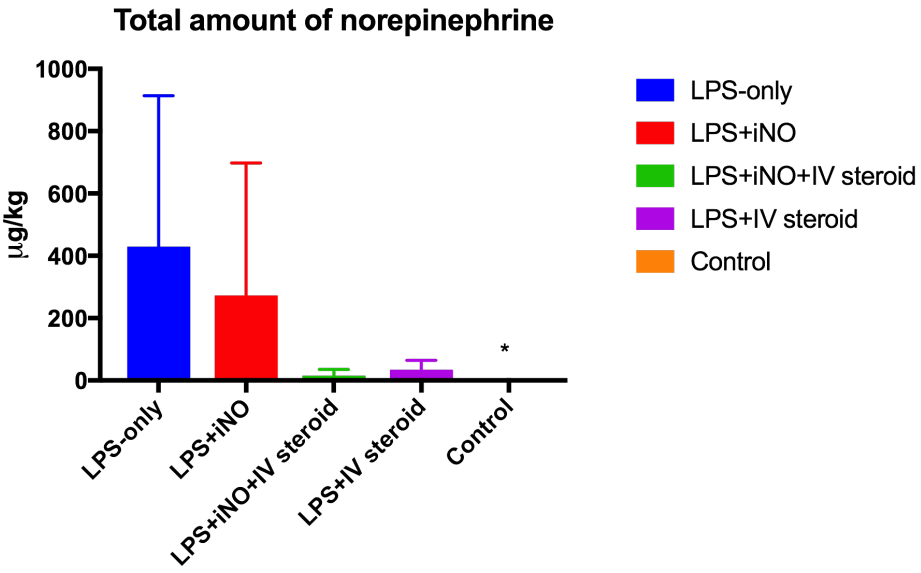

Figure 15. Total amount of norepinephrine administered over the study time in study I.

Norepinephrine consumption tended to be less in steroid treated groups (LPS + IV steroid and LPS + iNO + IV steroid) compared to LPS-only. Healthy control pigs were given significantly less norepinephrine ($p < 0.05$) compared to LPS-only.

LPS = lipopolysaccharide; iNO = inhaled nitric oxide; IV = intravenous; Control = sham animals, no LPS given; * $p < 0.05$ compared to LPS-only; Data are presented as median and IQR; N = 30 (6/group).

6.2.4 Pulmonary function

We could not see a significant difference in iNO treated groups (LPS + iNO and LPS + iNO + IV steroid) neither in mean pulmonary pressure (MPAP) (fig. 16A) nor in PaO2/FiO2 (fig. 16 B) compared to LPS-alone. However, there was a trend towards that iNO lowered the MPAP and increased the PaO2/FiO2.

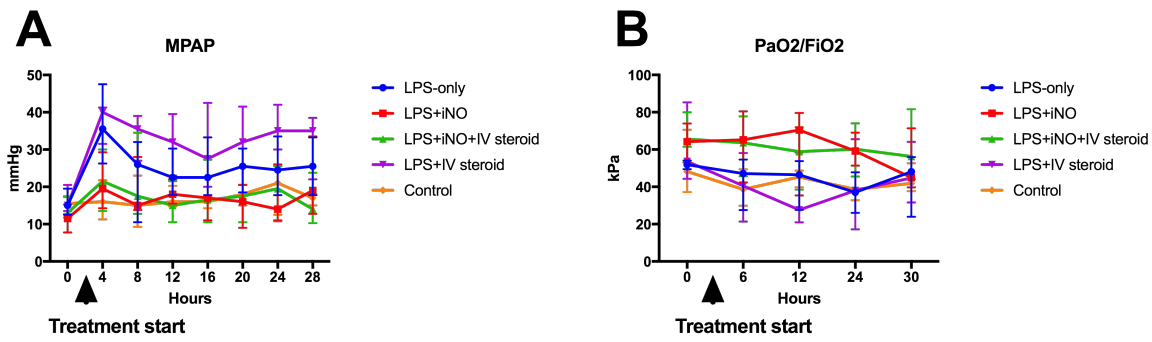

Figure 16. Treatment effect on pulmonary function

There was a trend towards a decreased MPAP and an increased PaO2/FiO2 in iNO-treated groups (LPS + iNO and LPS + iNO + IV Steroid) compared to LPS + IV Steroid and LPS-only.

LPS = Lipopolysaccharide; iNO = inhaled nitric oxide; IV = intravenous; Control = sham animals, no LPS given; kPa = kilo Pascal; mmHg = millimeter of mercury; time = change in in MPAP or PaO2/FiO2 over the study time; Data are presented as median and IQR; N = 30 (6/group).

6.3 VALIDATION OF A H3CIT ELISA (PAPER II)

An H3Cit ELISA in order to quantify levels of plasma H3Cit was methodologically validated.

The best-fit curve turned out to be a sigmoidal 4PL curve providing a **linear interval** of the curve with a range of ≈ 0.5 and 3.5 O.D., matching concentrations rangeing between ≈ 5 and ≈ 300 ng/mL.

In order to determine the **limit of detection**, the lowest detectable concentration was defined by approximation of the lowest detectable concentration obtained by the curve to ≈ 5 ng/mL, consistent with the 4PL match of the standard curve and the intersection of the lower asymptote of the upper 95% CI. The stated probability of the limit of detection was therefore set to approximately 5 ng/mL.

Three different batches of frozen H3Cit-standard, prepared on three different days and with different batches of PAD4 were compared for detector response, without significant

differences (fig. 17A). Similarly, no significant difference could be identified in the detection response obtained from a standard prepared from freshly citrullinated H3Cit contrasted to a standard prepared from frozen aliquots of H3Cit (fig. 17B), exposing a high stability and further allowing for high reproducibility.

The detection response of non-citrullinated histone H3 was considered negligible within the linear interval of the assay, despite a low detection response when identifying large quantities of non-citrullinated histone H3, verifying a high specificity for citrullinated H3 (H3Cit) within the linear interval of the assay (fig. 17C)

Recovery could not be obtained by adding/"spiking" known concentrations of H3Cit into plasma from healthy individuals (fig. 17D), probably indicating an "effect of the matrix" (in this case; plasma).

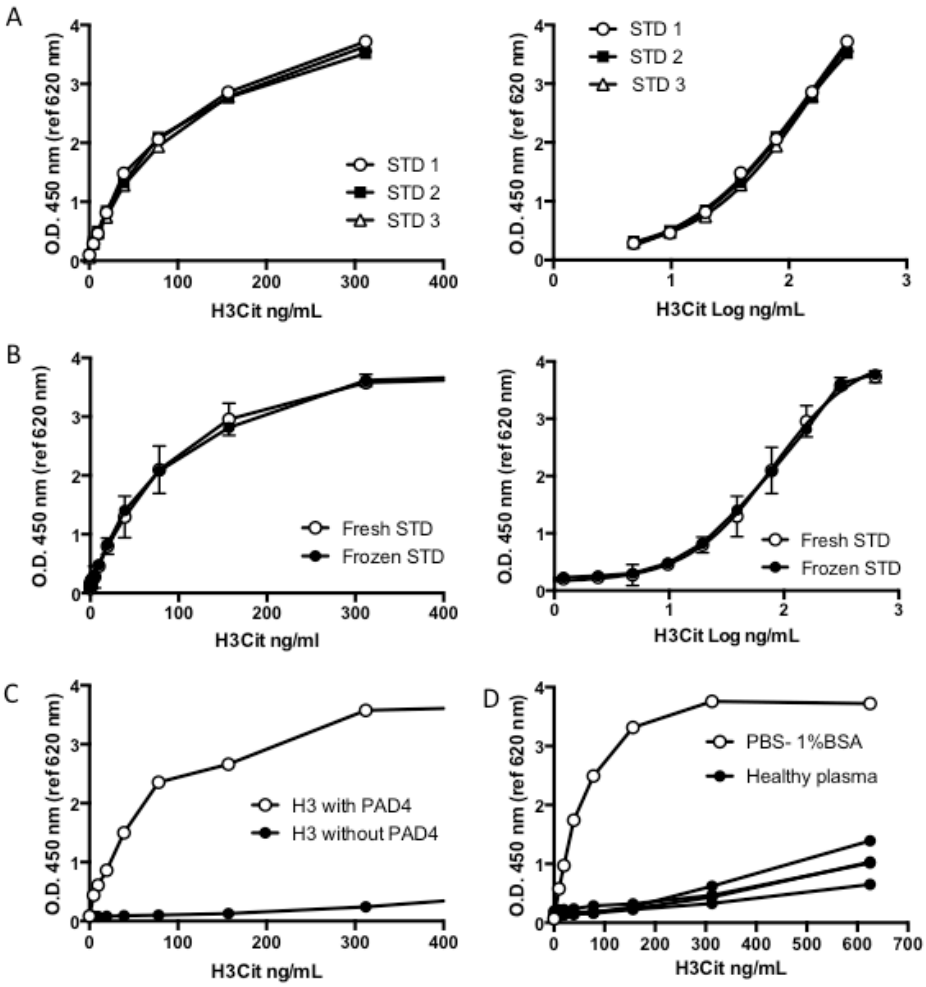

Figure 17. Stability and specificity. *Reprinted from Thålin, C., Daleskog, M., Göransson, S.P. et al. Immunol Res (2017) 65: 706. https://doi-org.proxy.kib.ki.se/10.1007/s12026-017-8905-3. © Thålin et al 2017, with permission under the terms of the Creative Commons Attribution License, open access.*

A) Illustrating similar detector responses of three standard curves prepared from three different batches of deep-frozen PAD4-citrullinated histone H3, or **B)** from frozen aliquots of H3Cit or freshly citrullinated H3Cit (F (DFn, DFd)= 2.6 (8,9); p= 0.088, and F (DFn, DFd)= 0.2 (4, 52); p= 0.916 respectively). **C)** Results acquired when preparing a standard curve with histone H3 that was incubated during the same circumstances as the standard preparation of H3Cit, but in lack of PAD4, resulting in non-citrullinated histones, illustrative of three different experiments. A low amount of antibody antigen was detected when large quantities of non-citrullinated histone H3 was added, but the antibody antigen detection was shown to be specific for H3Cit within the linear interval of the assay. **D)** No detectable H3Cit could be measured in plasma from healthy individuals. However, spiking of known concentrations of H3Cit into plasma diluted in proportions 1:2 yielded a significantly lower detector response when comparing with the detector response achieved from the standard prepared from PBS-1% BSA, implying an effect of the matrix.

H3Cit = Citrullinated Histone H3; PAD4 = Protein arginine deiminase 4; H3 = Histone 3; STD = Standard solution with known concentrations of H3Cit; PBS = Phosphate buffered saline.

In all samples taken at baseline, the levels of H3Cit were under the detection limit of 5 ng/mL. Further, in all samples taken from the very same individuals 3-4 h after LPS injection, H3Cit levels ranged between 28.7 ng/mL and 93.2 ng/mL. The CV were all <15 %, with an intra-assay variation ranging between 2.13-5.15 %. The inter-assay variation ranged from 5.80-12.55 %, indicating a high precision, as well as a good repeatability and reproducibility of the assay (table 5).

Sample	1	2	3	4	5	6	7	8
% CV Interassay (n=4)	11.54	10.27	12.55	8.5	9.6	10.53	5.8	13.5
% CV Intraassay (n=6)	5.1	4.5	5.08	2.7	4.35	3.58	2.13	3.1

Table 5. Precision; four different days inter-assay reproducibility and intra-assay repeatability. *Reprinted from Thålin, C., Daleskog, M., Göransson, S.P. et al. Immunol Res (2017) 65: 706. https://doi-org.proxy.kib.ki.se/10.1007/s12026-017-8905-3. © Thålin et al 2017, with permission under the terms of the Creative Commons Attribution License, open access.*

The H3Cit ELISA showed a high precision, with all inter- and intra-assay coefficients of variation (CV) < 15%.

6.4 CIRCULATING H3CIT IS ELEVATED IN A HUMAN MODEL OF ENDOTOXEMIA AND CAN BE DETECTED BOUND TO MICROVESICLES (PAPER III)

In this study rapid and significant elevations of circulating H3Cit following LPS injection in a human model of endotoxemia were reported. Results were obtained both by an ELISA and a flow cytometric assay, providing two distinct methods to quantify H3Cit in human plasma, both readily applicable in a clinical setting. Furthermore, for the first time, H3Cit was shown to be detectable bound to MVs. These results imply a novel mechanism by which H3Cit may be disseminated throughout the vasculature.

6.4.1 Circulating H3Cit levels are elevated after LPS injection in a human model of endotoxemia and can be measured both by ELISA and by flow cytometry.

The plasma levels of H3Cit were determined by ELISA and flow cytometry in a human model of endotoxemia (fig 18). H3Cit levels measured by ELISA displayed a five-fold increase in median plasma H3Cit levels 2 hrs after LPS injection ($p < 0.001$) compared to baseline, with a peak at 4 hrs ($p < 0.001$). The levels decreased between 4 hrs and 7 hrs post LPS injection ($p < 0.001$), but did not yet reach baseline (fig 18A).

To illustrate whether H3Cit can be measured bound to circulating MVs, we continued by performing a flow cytometric assay in order to determine H3Cit-bearing MVs in plasma. A slight but significant increase could be seen in H3Cit-bearing MVs 2 hrs post LPS injection ($p < 0.01$) and the median level rose 13-fold by 4 hrs ($p < 0.001$) compared to baseline. The increased levels withstood over the 7 hr study time (fig 18a).

No significant elevation was seen in the placebo arm neither by using the H3Cit ELISA nor the flow cytometry assay. The different dynamics found in the levels of MV-bound H3Cit detected by flow cytometry and circulating H3Cit detected by ELISA are shown in figure 18A, implying a late but sustained elevation of MV-bound H3Cit.

In order to assess the fraction of H3Cit bound to MVs in the samples, we continued with an ELISA-quantification of H3Cit in plasma post high-speed centrifugation, rendering an MV-enriched pellet and an MV-free supernatant. The level of H3Cit detected in MV-free supernatant was roughly 85% of the total amount of H3Cit found in plasma. The amount of H3Cit detected in the MV-enriched pellet, i.e. the portion assumed to be bound to MVs, accounted for around 10% of the total amount of H3Cit detected in plasma (fig 18B).

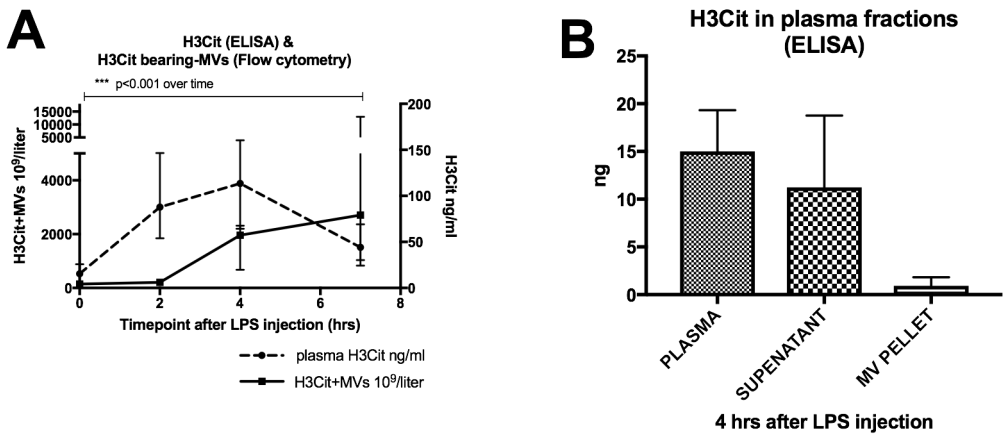

Figure 18. Circulating levels of H3Cit increased significantly after LPS injection in a human model of endotoxemia, comprising both an MV-bound and a non-MV-bound portion. Only levels of the LPS arm are plotted in this panel.

A) H3Cit-bearing MVs detected by flow cytometry (—) displayed a delayed but sustained increase compared to ELISA-detection of total plasma H3Cit levels (---).

B) ELISA-detected H3Cit levels in MV-free supernatant and MV-enriched pellet 4 hrs post LPS injection were approximately 85 % and 10 % respectively of the total amount of H3Cit detected in plasma.

H3Cit; citrullinated histone H3, MVs; microvesicles, ELISA; enzyme-linked immunosorbent assay, LPS; lipopolysaccharide, time; change in levels of H3Cit over the study time, groups; LPS versus placebo.

Data are presented as median and IQR. No significant difference was seen if LPS was administered at 1st or 2nd study occasion. N=22 for all observations

6.4.2 H3Cit binds selectively to MVs expressing PS in vitro

To study if H3Cit was able to bind to MVs and to further determine whether this binding is in part an attachment between phosfatedyl serine (PS) exposed on MVs and H3Cit, we proceeded to incubate *in- vitro* formed platelet-derived MVs from healthy donor-blood with purified H3Cit. By flow cytometry, MVs were first phenotyped based on PS exposure. The amount of H3Cit-bearing MVs in each population (PS negative MVs and PS positive MVs) was subsequently examined at every concentration of H3Cit incubation (0-1500 ng/ml). Results exhibited that H3Cit binds selectively to MVs that are PS positive in a concentration dependent way as illustrated in figure 19. No elevation could be seen in H3Cit-bearing MVs in the MV population negative for PS. Taken together, these findings indicate a binding/complex formation between PS exposed on platelet-derived MVs and H3Cit, conceivably through electrostatic forces between the negatively charged PS and the positively charged H3Cit.

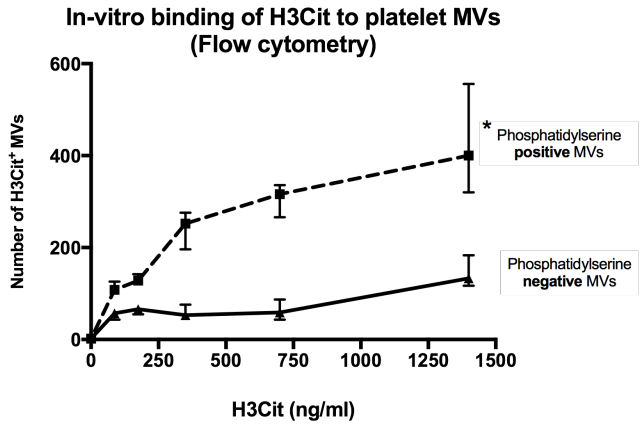

Figure 19. H3Cit binds selectively to MVs positive for PS

In-vitro generated PS negative and PS positive platelet-derived MVs were incubated in various concentrations of purified H3Cit. A significant increase in H3Cit-bearing MVs was only seen in the population of MVs positive for PS (* $p < 0.05$) when incubated in an increasing concentration of H3Cit. Results are illustrated as median and range (N=3). MVs = microvesicles, H3Cit =citrullinated histone H3, PS = phosfatidylserine. Data are presented as median and range.

6.4.3 Dynamics of phenotyped MVs after LPS injection

The levels of platelet-derived MVs increased and peaked prior to neutrophil-derived and H3Cit-bearing MVs as shown in figure 20. These dynamics of the MVs corroborate the current hypothesis that platelets and neutrophils interact during NETosis [116] in that platelets present LPS to the neutrophil in order to induce NETosis [40].

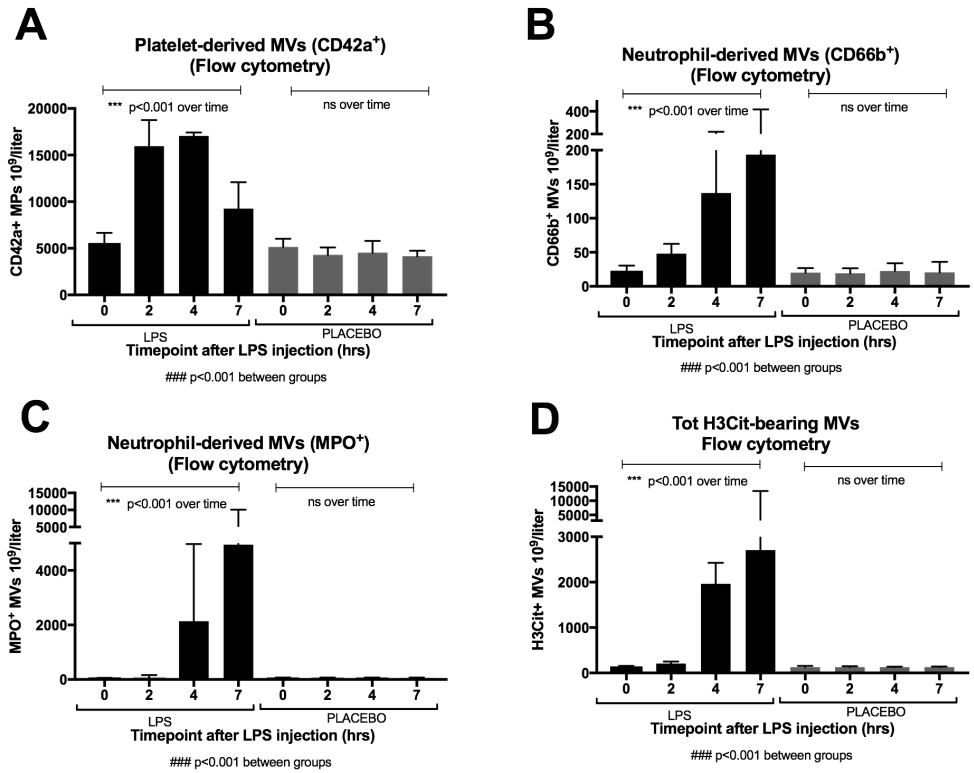

Figure 20. Levels of circulating platelet- and neutrophil-derived MVs, and H3Cit-bearing MVs after LPS injection

A) The number of platelet-derived MVs ($CD42a^+$MVs) peaked 2 hrs post LPS injection. B-D) In contrast, the number of neutrophil-derived MVs ($CD66b^+$MVs and MPO^+MVs) and H3Cit-bearing MVs peaked 4-7 hrs following LPS-injection. The placebo arm showed no significant dynamics over the study time. MVs; microvesicles; LPS; lipopolysaccharide; H3Cit; citrullinated histone H3, time; change in levels of MVs over the study time.

Data are presented as median and IQR. No significant difference was seen if LPS was administered at 1st or 2nd study occasion. N=22 for all observations.

Further we phenotyped H3Cit bearing MVs and found that they were derived from both platelets ($CD42a^+$) and neutrophils ($CD66b^+$ and MPO^+) with the most prominent increase in neutrophil derived H3Cit-bearing MVs, especially MPO^+MVs bearing H3Cit (fig 21).

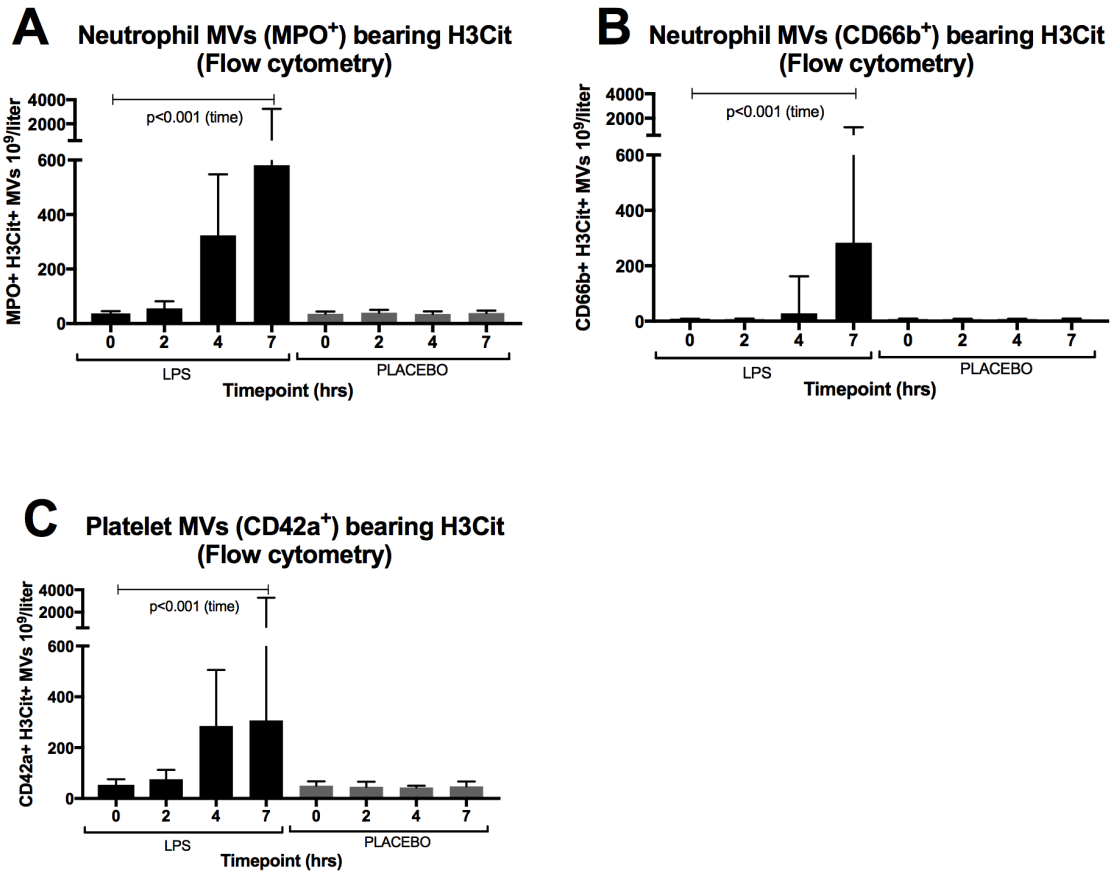

Figure 21. Phenotypic analyses of MVs indicated that H3Cit-bearing MVs were derived from both platelets and neutrophils

H3Cit-bearing MVs exposed both neutrophil (MPO; **A)** and CD66b; **B)** and platelet (CD42a; **C)** specific markers, with the most prominent increases in H3Cit-bearing MVs exposing MPO.

H3Cit; citrullinated histone H3, MVs; microvesicles, LPS; lipopolysaccharide, time; change in levels of MVs over the study time; groups; LPS versus placebo.

Data are presented as median and IQR. No significant difference was seen if LPS was administered at 1st or 2nd study occasion. N=22 for all observations.

7 DISCUSSION

Within this thesis we found that iNO+steroid treatment tended to have an organ protective effect, mainly in the kidney, although we did not find any grand significances. Moreover, we validated an ELISA assay quantifying H3Cit, a biomarker that has revealed great interest in the context of sepsis, in human plasma. Using this ELISA assay, we proceeded to demonstrate elevations in plasma H3Cit after LPS injection in a human model of endotoxemia, supportive of the potential role of H3Cit in sepsis. We furthermore report novel data on MV-bound H3Cit, quantifiable by flow cytometry. This not only allows for a rapid and robust assessment of in vivo NET formation in a large number of samples, but also proposes a possible mechanism by which NET components, such as H3Cit, may be disseminated throughout the vasculature. ELISA and flow cytometry are two clinically applicable methods that could aid in further research investigating the importance of H3Cit as a biomarker in sepsis. Following is a discussion about possible implications of our findings.

7.1 QUANTIFICATION OF H3CIT

Despite an emerging interest in the field of NETs, there is still a lack of a golden standard method or marker to distinguish and quantify NETs in plasma. Consequently, a number of markers and methods have been used to establish the presence of NETs [60]. The differences in methodologies make it difficult to compare and to interpret results between studies. Moreover, as discussed previously, some of these methods are impeded by lack of objectivity, quantification and specificity. Paper II proposes an assay allowing for a fast, highly objective, specific and reliable quantification of the NET-specific marker H3Cit in human plasma. This assay denotes a first step towards a consistency and a specific, as well as an objective quantification of NETs in human plasma.

Although the validation of the H3Cit ELISA displayed a high specificity and reproducibility, supported by recent papers implementing the assay [117, 118], there are certain limitations of the validation procedure worth noting. Trueness of the concentrations of H3Cit detected in plasma could not be assessed in the absence of an established method for comparison or a reference analyte of known concentration. Furthermore, the concentration of H3Cit in the standard curve is an approximation based on an optimal enzymatic activity of PAD4, assuming that all histones are citrullinated. An underestimation of H3Cit-concentrations in the samples tested can thereby not be ruled out. Moreover, known concentrations of H3Cit spiked in plasma from healthy donors could not be recovered. Presumably, this was due to the rapid degradation of free histones in plasma (half-life of 4,6min) [119]. Opposed to free histones in plasma, H3Cit quantified in our samples are assumed to be protected by surrounding DNA strands, as part of a nucleosome within a NET complex and/or by bondage to other protein structures such as MVs. It is thus possible that the H3Cit quantified by the H3Cit ELISA constitutes the amount of H3Cit sheltered by the NET complex or other protein structures, excluding a potential portion of free H3Cit in plasma. Even though the portion of

H3Cit attached to DNA strands in NET complexes is the main point of interest when quantifying NETs, the effect of the matrix, however, does impose a limitation to this assay validation.

However, despite these limitations, the methodological validation showed a high specificity for H3Cit and allows for a good reproducibility, with CVs all < 15% due to a high stability of the custom-made standard.

We show that H3Cit can bind to MVs, rendering flow cytometry a feasible method for quantifying H3Cit. Although we cannot rule out an *in vitro* electrostatic binding between MVs and H3Cit, a recent study by Wang et al [120] indeed report an *in vivo* complex formation between MVs and NETs. This study shows that MVs bind to NETs by means of PS on the surface of MVs. Furthermore, the complex of NETs-MVs promoted thrombin generation as opposed to NETs not carrying MVs, suggesting that MVs play a critical role in NET-induced thrombin generation. Speculating further, MVs may bind to H3Cit entangled in NET-complexes, and the MV-H3Cit complex may subsequently detach and be transported further downstream. As mentioned before, the portion of circulating H3Cit-bearing MVs quantified by flow cytometry in paper III, rose later and remained elevated longer as opposed to the total amount of circulating H3Cit, of which the largest part is assumed to be NET-bound, supporting this hypothesis. H3Cit carried by MVs may thus have a prolonged lifetime due to a protective effect of MV-binding after a dismantling of the NET scaffold. Interestingly, Ekaney et al [119] demonstrated that histone degradation reached a plateau after 30 min, with no further decline during the 6 hr observation. The authors speculate that the biological half-life of histones in plasma may be affected by the binding of histone fragments to other proteins protecting them from further degradation. MVs may thus serve as possible protecting binding proteins. The biological consequences of the H3Cit-MV complex remain to be clarified.

Others have measured NET-components by the means of flow cytometry [121, 122]. These studies detect neutrophils positive for NET- associated components. The nature of neutrophils though, may pose a potential problem, as they tend to form aggregates making them difficult to distinguish as single cells in the flow cytometer [123]. Furthermore, image-based flow cytometry, detecting the elongating shape of a decondensed nucleus as a sign of NETosis has been proposed [124]. Future studies will determine whether the H3Cit ELISA and/or the flow cytometry assay for H3Cit-bearing MVs, could be helpful in a clinical setting determining a systemic NET formation in various diseases, such as sepsis.

7.2 EFFECTS OF INO + IV STEROID IN ENDOTOXEMIA

We could not reproduce the successful data from Da et al in attenuating the multi-organ failure in lung, kidney and liver by combining iNO + IV steroid [83], in a similar porcine endotoxin model extended from 6 to 30 hrs. One reason could be a lack of power, given the greater spread in the data, compared to the study by Da et al. Nevertheless, in spite of the

absence of statistical significance, a trend towards a beneficial effect for iNO + IV steroid treatment compared to single treatments, was seen for most variables.

Firstly, there were no deaths in the LPS + iNO+ steroid group as opposed to other LPS treated groups, corroborating a protective role of iNO+ steroid treatment. Secondly, there was a tendency toward attenuated renal failure, exemplified by the significantly better/higher base excess after LPS + iNO + IV steroid treatment compared to after LPS infusion only, indicating a preserved buffering function in the kidney. Interestingly, this finding was apparent in spite of administration of HES, an IV fluid composed of starch that has since the study was performed, been reported to induce renal failure [125]. HES was given to all animals receiving LPS with no significant difference in volume between groups. Our data signaling a beneficial trend in iNO+steroid attenuation of renal failure is supported by a recent publication using a porcine ischemia reperfusion model, reporting that iNO+steroid treatment had a beneficial effect by down regulation of renal TLR4 mRNA expression [126]. Moreover, iNO has been reported to increase renal filtration [127]. Conversely, iNO treated patients were reported to have higher incidence of renal failure in a Cochrane review [128].

Thirdly, steroid treated groups (LPS + IV steroid and LPS + iNO + IV steroid) tended to require less norepinephrine compared to the other LPS treated groups (LPS-only and LPS + iNO). This effect could be explained by the enhanced pressor response to norepinephrine obtained after administration of steroid in sepsis [108]. Interestingly, the LPS + iNO + IV steroid treated pigs only required norepinephrine before treatment was initiated, as opposed to the LPS + IV steroid group. This increased hemodynamic stability in the LPS + iNO + IV steroid group, could possibly be explained by the LPS-induced down regulation of the glucocorticoid receptor [83], which is counteracted by the up regulation of the very same receptor by iNO in the LPS + iNO + IV steroid group, a mechanism proposed by Da et al [83].

INO is known to decrease pulmonary artery hypertension by selective dilatation of pulmonary vessels in ventilated areas, thereby increasing the PaO2/FiO2 ratio [98, 99]. We did not see a significant improvement in neither of these parameters by administering 30 ppm iNO. This could possibly be explained by the fact that some LPS-treated animals did not exhibit a PaO2/FiO2 of less than 40 kPa, which is one criterion for an Acute Respiratory Distress Syndrome (ARDS) diagnosis [129] and suggest an absence of shunting in the lung, thereby leaving little room for iNO to exert its effect. Also, some individuals with pulmonary hypertension have been reported to be non-responders to iNO, defined as failure in obtaining an improved oxygen tension of at least 20% and/or a decrease in mean pulmonary artery pressure of a minimum of 15% [130]. However, a trend towards a decreased MPAP was seen in iNO treated groups.

The iNO treatment in study I aimed primarily at an extra-pulmonary anti-inflammatory effect. In ARDS it has been shown that a lower dose of iNO up to 10 ppm, gives a maximally improved oxygenation, which again worsens as the dose increases [131]. This is in contrast to the dilatory effect on the pulmonary circulation, which is enhanced as the dose is increased.

Thus, a controversy appeared at that time: which pharmacodynamic effect should be desired for better outcome - best oxygenation or lowest MPAP? In similar, we do not yet know for sure which dose of iNO to use in order to reach desired extra-pulmonary effects. Previous data reported peripheral effects after administration of iNO 80 ppm during regional NO-blockade on increased peripheral NO delivery, by nitrosithols [132] and on improved intestinal blood flow after ischemia/reperfusion (I/R) in a feline model [133]. Moreover, 40 ppm iNO, a dose that has been reported to be safe and well-tolerated [134], increased renal filtration [127]. The precise dose of iNO 30 ppm, was chosen in accordance with Da et al, as was the steroid dose administered, in order to obtain a similar organ preserving effect from iNO + IV steroid reported after 3 hrs of LPS infusion [83]. Given the longer study time in study I, the 75 mg steroid dose was repeated every 8 hrs, rendering a steroid dose of 9-15 mg/kg/day, for the pigs included. The steroid dose administered could be compared to the proposal from the "surviving sepsis campaign", recommending a hydrocortisone dose of not more than ≈ 3mg/kg/day for vasopressor resistant septic shock [17].

Finally, the choice of an endotoxin model instead of a real sepsis model is worth discussing: Once and again endotoxin was chosen in analogy with Da et al [83] since we wanted to see if their results were reproducible even after 30 hrs of LPS infusion, which better agree with the clinical reality. However, knowing that endotoxin should rather be considered a model of inflammation and not sepsis, we also intended to examine the combination of iNO+steroid on another possibly more clinically relevant sepsis model, cecal ligature and puncture (CLP) [97]. In the CLP model, the caecum is perforated in anesthetized pigs [135]. However, perforation alone did not produce septic shock in our model. Therefore a ligation of the mesenteric superior artery producing an I/R reaction was carried out, like in a model earlier described [136]. Unfortunately, in our hands, this model proved to be extremely unreliable. Indeed, some pigs developed septic shock while others only succumbed to a SIRS reaction. This problem has also been described by others [80]. In an article using CLP on a mini-pig model the authors discuss that the grade of sepsis could be due to the size of the caecum perforation [137]. Possibly, our cecal puncture was too small. We have chosen to report our failure to induce a septic shock by CLP and ligation as a short report (yet to be published).

7.3 H3CIT AS A POSSIBLE BIOMARKER EVALUATING TREATMENTS FOR MOF?

The choice of using biomarkers for single organs as outcome variables in paper I, could be discussed. Considering that septic shock is a disease affecting the whole body, it would probably have been wise to use an outcome variable measuring multiple organ failure. Up to date, such a biomarker does not exist, but the Sequential Organ Failure Assessment (SOFA) score [21] has been frequently used. However, SOFA score is validated for humans and includes the Glasgow Coma Scale (GCS), requiring verbal ability. Consequently SOFA score is not applicable to anesthetized pigs, even though it has been used when evaluating organ failure in awake pigs [138] and dogs [139]. Modified to evaluate reaction to sounds rather

than classic GCS verbal criteria's, SOFA score seemed to predict mortality also in animals. However, a recently validated organ failure score for specific use in critically ill horses, performed better in predicting 6-month survival from discharge, compared to scores extrapolated from humans [140], indicating a benefit of species-specific organ failure scores. In the future it could be worth considering the use of a validated organ score for sedated pigs that are frequently used in medical research, in order to assess total organ dysfunction. Alternatively, it would be interesting to find a biomarker correlated to MOF that could be used as an outcome variable.

Several studies corroborate the association between high levels of circulating NET components, including histones, and their detrimental effects on the host. Components of NETs have been associated to activated coagulation [141] and chromatin decondensation mediated by PAD4, rendering H3Cit, has been implicated to be vital for thrombus formation [142]. Extracellular histones have further been showed to contribute to both micro- and macrovascular thrombosis, as well as endothelial and epithelial dysfunction, organ failure and tissue injury in murine models of sepsis [44, 45, 143, 144]. These data motivate further research on the potential of NET markers, such as H3Cit, as both sepsis-predictive biomarkers, as well as possible outcome biomarkers when evaluating treatments in an endotoxemic model or septic conditions involving NETosis.

Notably, H3Cit was not measured in study I. At the time for these experiments, the knowledge of H3Cit was not as vast as it is today. Therefore, it is yet to be established if iNO or steroid affect H3Cit levels. However, there could be a theoretical possibility that these drugs do diminish H3Cit levels since they inhibit the production of e.g. TNF-α and IL-8, two known triggers of NETosis [37, 145]. Furthermore, histones [77], as well as MVs [71], activate the NFκβ pathway, shown to be inhibited by iNO [105]. INO has further been reported to attenuate apoptosis in a rabbit ischemia-reperfusion model [146]. Interestingly, this study from 2004 used a technique to detect apoptosis identified as DNA strands on cells, and these cells were interpreted as apoptotic. The discovery of NETs may rather propose a presence of NETosis.

It could be speculated whether the drop in leukocyte count 1 hr post LPS injection in the human endotoxin model, as well as other studies [94] could reflect NETosis. The same drop in white blood cell count was seen in study I, noted 6 hrs after LPS infusion. TNF-α, a known trigger of NETosis [145], rising simultaneously with this drop in both the animal- and the human endotoxemia-model, corroborates this hypothesis. Interestingly, this drop in white blood cell count was more pronounced for the LPS-only and LPS + IV steroid group than in the LPS + iNO group and LPS + iNO + IV steroid, conceivably supporting the theory that iNO could inhibit NET formation. In addition, steroids have been reported to attenuate both I/R-induced kidney injury and sepsis through various mechanisms, comprising glucocorticoid receptor activation and protection [147], conceivably enhancing a potential protective effect of iNO.

Although speculative, H3Cit may be a reasonable outcome biomarker evaluating the effects by iNO + IV steroid on MOF in a future endotoxemia study.

7.4 H3CIT AS A TARGET FOR INTERVENTION

In light of the detrimental effects of NETs, promising NET inhibiting agents, such as PAD-inhibitors [64, 148, 149] and NET degrading agents such as DNase [45, 150, 151] have been evaluated in murine models, appearing to suppress circulating H3Cit levels with improved survival [148, 149, 152].

Up to date, it has not been sufficiently validated what would be most beneficial; to decompose existing NETs, or to inhibit NETosis. It is however, conceivable that the choice of drug could be dependent of timing during sepsis progression. Consequently, a PAD4 inhibitor could slow down the NET formation at an early stage, whereas DNase could possibly be useful at a later state when NETs are already abundant.

Notably, several authors have discussed the balance between the antimicrobial and cytotoxic effects of NETs as a preservation of NETosis, and possible favorable effects thereof in the early stage of sepsis may be necessary to combat the infection [45, 153]. Reflecting this hypothesis, it could be of value to determine a "cut-off" level for when NETs become detrimental and could benefit from being inhibited. ELISA- and/or flow cytometry quantification of circulating H3Cit, could be useful methods in deciding when to intervene with NET production or to follow disease progression. Interestingly, Biron et al found that PAD4 deficient mice had impaired NET formation, despite similar amount of neutrophil infiltration, in kidney and lung. These PAD4-deficient mice unable to release NETs, also displayed preserved renal function compared to wild type (WT) mice [154]. Furthermore, another study using PAD4 deficient mice, showed that these mice fared better compared to WT mice after infection with influenza A despite the absence of NET formation, suggesting that NET formation is dispensable for immunity against influenza infection [155]. In the same manner, Martinod et al showed that PAD4 deficient mice subjected to polymicrobial sepsis had comparable survival to WT mice. Interestingly, PAD4-deficient mice were further partially protected from LPS-induced shock with a reduced inflammatory and hypercoagulable/ prothrombotic state, compared to PAD4 WT mice [156]. Thereby, it can be speculated if NET function even at an early stage of sepsis and endotoxemia, is redundant.

In study I, iNO + IV steroid treatment was initiated when endotoxemia had already been persistent for 3 hrs. The time point for treatment start was decided in analogy with the study from Da et al [83] and was chosen in order to mimic the clinical reality in which sepsis patients present to the ICU after the onset of septic shock. Considering the hypothesis that iNO + steroid inhibit NETosis rather than degrade already existing NETs, perhaps a better effect could have been achieved if iNO+steroid was initiated earlier, possibly even before endotoxin infusion. The idea of prophylactically administrated iNO for better anti-inflammatory outcome is corroborated by an I/R model of anesthetized patients undergoing

knee surgery. Patients exposed to iNO at 80 ppm 30 min before and during tourniquet application, displayed significantly reduced inflammation [100]. The reduced inflammation could however not be shown in a similar study, in patients receiving spinal anesthesia, due to lack of inflammatory response [157]. Lang et al showed that preoperative administration of 80 ppm iNO improved liver function after transplantation and also decreased hospital length of stay [158]. The same dose given before and during myocardial ischemia and reperfusion in pigs, significantly reduced infarction size and improved microvascular perfusion, decreased leukocyte infiltration and decreased cardiomyocyte apoptosis [159]. Further, prophylactically administered iNO 80 ppm + IV steroid also showed a beneficial effect in an I/R model in pigs by diminishing the expression of messenger RNA TLR4 in kidney [126]. On the other hand, Da et al found that iNO + IV steroid had an organ protecting effect despite being administered 3 hrs post start of LPS infusion.

One hypothesis to the success of Da et al despite a relatively late administration of iNO+steroid, could be that LPS, being a potent trigger of NETosis [37], induces a continuous NETosis when given as an infusion until a possible rate-limiting step, as discussed in a previous article [153]. The authors propose that a limiting step in NETosis could be platelet depletion. In our porcine endotoxin model we found a significant fall in platelets after 24 hrs compared to baseline, possibly resulting in a decreased NET formation around this time point, which would be conceivable considering the pivotal role of platelets in LPS-induced NETosis [40]. The fall in platelet count for the healthy control group, could be due to the precence of the pulmonary arthery catheter (PAC). The use of PAC and the association to thrombocytopenia has been reported previously and is believed to be due to an increased consumption of platelets [160, 161] after microaggregation on the catheter with a subsequent phagocytosis by macrophages [162].

Interestingly, in the human model of endotoxemia, plasma levels of TNF-α peaked 1.5 hrs post LPS injection, whereas plasma H3Cit levels detected by ELISA peaked 3 hrs post LPS injection. TNF-α has been shown to trigger NETosis, and displayed a later rise at 6 hrs post LPS infusion in the porcine model of endotoxemia. A hypothetical NET burden may therefore have occurred later in the porcine model of endotoxemia and thus partly have contributed to multi-organ damage 12 hrs post LPS infusion. This hypothesis is supported by the increase in creatinine seen at this time point.

The potential of NET inhibitors to alleviate damaging NET-induced effects in endotoxemia and sepsis remains to be elucidated, as does the optimal balance between the antimicrobial and cytotoxic effects of NETs.

7.5 H3CIT AS A BIOMARKER FOR DIAGNOSES OF SEPTIC SHOCK

In the clinical setting of the emergency room or the intensive care unit, the appearance of low blood pressure could be a question of debate. Differential diagnoses could be heart failure, dehydration, hemorrhage, septic shock or possibly deep sedation among others. Although

H3Cit levels have been shown to be elevated in non-infectious conditions such as cancer [117] and thromboembolism [163], these diagnoses rarely go with shock. Studies have shown that H3Cit levels are not elevated in non-septic shock states, but in endotoxic and septic shock [149, 164], thereby making the use of H3Cit a possible diagnosing tool for septic shock.

Nevertheless, the specific pathogen causing the infection may be crucial in selecting a suitable biomarker to diagnose sepsis, since different pathogens could affect the immune system differently [165]. H3Cit may be a promising biomarker for sepsis caused by pathogens described to induce NET formation, such as E. coli, Staphylococcus aureus, Group A streptococci and Candida albicans [37], but it remains elusive whether all microbes induce NET formation. Consequently, H3Cit as well as other NET-components, could be pathogen-dependent biomarkers, and their role in a universal sepsis-screening setting is yet to be established

7.6 ROLE OF H3CIT AS A PREDICTIVE BIOMARKER FOR SEPSIS

As stated above, increased levels of H3Cit and other NET-components have been correlated to a higher SOFA score in a cross-sectional study of sepsis patients [45]. However it remains elusive if plasma levels of H3Cit could actually *predict* the severity of MOF associated with septic shock. Early elevations of plasma cfDNA have been detected at admission in trauma [166] and burn patients [167]. These levels increased further in patients developing septic shock including MOF. However, considering that cfDNA is elevated in tissue damage [61], it is not clear if the early rise in circulating cfDNA in these studies reflect NETosis or rather cellular damage from the trauma. In the study of multiple trauma patients by Margraf et al, high levels of cfDNA were associated with subsequent sepsis, MOF and death, but the origin of cfDNA can still be debated. Interestingly, the results from Hampson et al [164], showed early rises in cfDNA in burn patients, as opposed to plasma H3Cit detected by western blot, which rose only during septic episodes thereafter. These data might suggest that H3Cit may be a more specific marker for predicting a second hit with sepsis compared to cfDNA, in burn and possibly trauma patients.

Pan et al showed elevated plasma H3Cit levels as early as 30 min after LPS injection. Moreover, H3Cit was found to be more reactive to endotoxemia than the inflammatory cytokines IL-1β and IL-6 as well as the clinically employed septic biomarker procalcitonin [149]. CRP also increased relatively late, at 6 hrs after endotoxemia injection in humans [94], which was the reason of not measuring CRP in our 7 hrs human endotoxemia model. These findings could suggest that H3Cit might have a place in predicting sepsis before the onset of symptoms, as well as before increased levels of procalcitonin and CRP. Septic shock may develop quickly urging the need for early and reliable biomarkers. Flow cytometry may, in this respect be favorable over ELISA methodology, rendering quick results within the hour, as opposed to the ELISA assay, which takes several hours. Notably, our first sample analysed

for H3Cit after LPS injection was at 2 hours in the human endotoxin model of paper III, i.e. *after* the onset of clinical symptoms, which appeared 1hr post LPS injection, limiting our ability to assess plasma H3Cit as an early blood biomarker. The levels of $H3Cit^{+}$MVs, although significantly elevated at 2 hrs compared to baseline, did however, not show the same dramatic early elevation as H3Cit measured with ELISA at the same time point, indicating that MV-bound H3Cit may rise later than H3Cit anlysed by ELISA, that could possibly be NET-bound.

Delabranche et al have suggested endothelial derived MVs as a predictive biomarker of disseminated intravascular coagulation (DIC) [168]. The authors speculate about MVs as a more clinically relevant biomarker of early DIC, being more easily measured when circulating in blood compared to NETs that could be trapped in thrombi. Our data illustrating that the peak of H3cit measured by ELISA precedes the predominant levels of MVs, might contradict this theory. Further, the clear rise in platelet-derived ($CD42^{+}$) MVs, preceding the peak of H3Cit-bearing MVs in paper III, raises the question as to whether platelet- derived MVs may be an earlier biomarker, possibly mirroring a subsequent NET-formation. This hypothesis is corroborated by the role of platelets in NETosis [40] and is further supported by data reporting that a decreased septic-release of MVs by inhibition of the NFκβ pathway, resulted in attenuated inflammatory- and DIC processes, as well as organ dysfunction, and an increased survival after CLP induced sepsis [73]. Nevertheless, it remains elusive whether the NFκβ-inhibition also affects NETosis directly. In contrast, Soriano et al showed that platelet and leukocyte derived MVs were inversely correlated with SOFA score [71]. Inconsistencies concerning the role of MVs in sepsis could possibly be explained by different profile-variations of MVs depending of the source of infection [165], which corroborate the above-discussed hypothesis about pathogen specific biomarkers.

7.7 PROBLEMS AND OTHER FUTURE APPROACHES OF BIOMARKERS

The theory about different pathogen dependent immune-profiles reinforces the commonly discussed thesis that sepsis cannot be predicted by one single-, but rather a combination of different biomarkers. Conceivably, H3Cit could play a role in such a battery. Finding new reliable biomarkers for septic shock associated with outcome could not only predict the disease course of sepsis, but also advance the medical research in evaluating new treatments. At the same time, one has to be humble before the complexity of the sepsis pathogenesis. Targeting one single biomarker may not be a viable approach for treating an intricate inflammatory response to diverse pathogens. Similarly, timing might be crucial considering that cytokines and immune cells play both protective and pathogenic roles in sepsis.

Lately, so called Omics, combining quantifications of e.g. transcription factors like messenger RNA, that translate into different molecules included in the pathogeneses of sepsis have rendered some interest. Omics have been studied in order to predict the outcome of sepsis [15]. Speculating widely, the Omics technique could possibly be an earlier way to

detect deleterious components in the sepsis pathology, conceivably making them possible to target before the actual NET formation. Considering the genetic predisposition in developing septic shock, it could further be speculated if a certain genetic defect could be detected by Omics, thereby possibly identifying individuals that experience a higher risk for sepsis with MOF.

Conclusively, summing up the results from this thesis, as well as other reports from the literature, circulating H3Cit may have a potential role as a biomarker in endotoxemia and sepsis, both by diagnosing as well as predicting outcome. However, in order to decide the value of circulating H3Cit as a biomarker in sepsis, further research is warranted in real-life clinical settings. For this purpose, the H3Cit ELISA presented in paper II and the flow cytometric assay detecting H3Cit-bearing MVs presented in paper III may be helpful. Moreover, further research will reveal if H3Cit could be a target of intervention in the future, possibly even by iNO. Meanwhile, it would be interesting to validate H3Cit as a marker of MOF in endotoxemia- or other sepsis models, when studying potential sepsis treatments.

8 CONCLUSIONS

- **A combination of iNO+steroid tends to attenutate the multi-organ failure** associated with endotoxin stimulation in pigs, although no clear significances between LPS + iNO + IV steroid compared to LPS-only could be proven in our material. **(Paper I)**

- In order to prove significances **when studying MOF** in endotoxic and possibly septic shock, **an outcome varible weighing in the total organ failure should be used,** rather than just one single organ specific biomarker (e.g. creatinine, blood pressure, mean pulmonary artery pressure, platelets, base excess). As such, scoring systems like SOFA score apply. **(Paper I)**

- Levels of **H3Cit,** a NET specific marker, **can be reliably quantified** in human plasma **by** means of a novel enzyme-linked immunosorbent assay (**ELISA**), allowing for a high precision, stability and specificity. **(Paper II)**

- **Circulating H3Cit is elevated in humans after endotoxin injection and can be detected by both an ELISA and bound to microvesicles (MVs), by flow cytometr**y. The MV-bound portion of H3Cit seems to remain elevated longer than the ELISA-detected portion, assumed to be bound to NETs. This suggests that H3Cit may be disseminated throughout the vasculature bound to, and possibly protected, by MVs. **(Paper III)**

9 SVENSK SAMMANFATTNING

9.1 BAKGRUND

Den här avhandlingen behandlar ämnet svår blodförgiftning, som i vissa fall kan drabba hela kroppen genom kraftig inflammation. Det ger upphov till organsvikt. Tillståndet har en hög dödlighet på ca 25-30%, som kan minskas förutsatt att diagnosen ställs tidigt i kombination med att rätt behandling, t.ex. antibiotika och intensivvård kan sättas in snabbt. Svår blodförgiftning är inte lätt att diagnostisera korrekt och tidigt, då vi idag inte har specifika blodprover för att fastställa sjukdomen. Troligen skulle man också kunna sänka dödligheten i svår blodförgiftning om det gick att minska den organsvikt som sjukdomen medför. Någon medicin för att minska organsvikten finns inte idag.

9.2 SYFTEN

I första delarbetet ville vi **studera om** två i Sverige redan registrerade läkemedel, **inhalerat kväveoxid (iNO) och kortison** i kombination, **kan minska graden av organsvikt** som uppkommer i samband med svår sepsis. Vidare ville vi **mäta hur proteinet citrullinerat histon H3 (H3Cit) i blodet reagerar vid inflammation** (delarbete 3). H3Cit har de senaste åren rapporterats vara en blodmarkör som man skulle kunna mäta, både för att eventuellt kunna avgöra vem som kommer drabbas av svår blodförgiftning, samt för att kunna ställa diagnosen svår blodförgiftning. Det finns dessutom data i litteraturen som indikerar att H3Cit i sig har en ”giftverkan” och att behandling inriktad på att hämma nivåerna av H3Cit i blodet skulle kunna minska skadliga effekter av blodförgiftning på kroppen. H3Cit har däremot varit svårstuderat eftersom det inte funnits någon bevisat fungerande metod för att mäta H3Cit i blodet. För att kunna studera H3Cit på ett bra sätt utvecklades därför först en metod för att kunna mäta det i human plasma (delarbete 2). För att bättre förstå vilken betydelse H3Cit har för sjukdomsförloppet vid blodförgiftning, är det viktigt att **veta på vilket sätt H3Cit cirkulerar i kroppen.** Vi ville därför se om det kunde vara bundet till en annan partikel i blodet s.k. mikrovesiklar (delarbete 3), som har visats kunna transportera olika former av molekyler genom kroppen. Mikrovesiklar är som ett ”kladdigt damm” som frisätts vid blodförgiftning bl.a. från immunförsvarets celler.

9.3 METODER

Utvärderingen av iNO + kortisons effekt gjordes på **sövda grisar** eftersom man inte får studera nya behandlingar direkt på människor. Metoden för att mäta H3Cit är en ELISA-baserad metod, och utvecklades i enighet med bestämda riktlinjer. **ELISA**n användes sedan för att se hur H3Cit rör sig över tid vid inflammation. Detta möjliggjordes genom att ett övergående, lättare inflammations-tillstånd (feber, muskel- och huvudvärk) skapades hos

friska försökspersoner. För att mäta om H3Cit kunde binda till mikrovesiklar användes metoden **flödescytometri.**

Forskningen i avhandlingsarbetet är experimentell. Experimentell forskning är viktig eftersom patienter med blodförgiftning inom sjukvården utgör en mycket blandad grupp. Patienterna kommer t.ex. till sjukhus i olika sjukdomsstadier och är infekterade med olika bakterier, bara för att nämna några faktorer. Via experimentell forskning kan också kroppens svar på inflammation studeras på ett mer standardiserat sätt. På så vis blir det lättare att tolka de provsvar man får från sjuka patienter i framtida studier.

9.4 RESULTAT

Vi hittade inte statistiskt signifikanta skillnader med mindre organsvikt för behandlingsgruppen som fått iNO + kortison jämfört med de sjuka grisar som inte fått behandling. Däremot kunde man se **en tendens till bl.a. minskad njurskada hos iNO+kortison gruppen.** Vidare **kunde cirkulerande H3Cit nivåer** hos människa med lättare inflammations-tillstånd **mätas med den i delarbete 2 utvecklade ELISA metoden.** Med flödescytometri kunde vi **även påvisa att H3Cit binder till mikrovesiklar**

9.5 SLUTSATSER

INO+kortison visar en tendens till att skydda kroppens organ vid den svåra inflammation som sker vid blodförgiftning i studien på sövda grisar. Kunskap om mer exakta verkningsmekanismer och optimala doser av dessa läkemedel är däremot fortsatt bristfällig. Skyddseffekterna av kombinationsbehandlingen iNO + kortison är för svaga för att på basen av fynden i den här avhandlingen föreslå behandlingsförsök på människa med blodförgiftning. Vidare så **kan H3Cit,** vilket föreslagits som en viktig parameter vid svår sepsis, **mätas tillförlitligt i blod med två oberoende metoder** vilka båda är möjliga att använda i klinisk praxis. Våra resultat talar också för att **H3Cit kan transporteras i blodcirkulationen bundet till mikrovesikler.** Betydelsen av mikrovesikelbundet H3Cit är däremot inte klarlagd.

10 ACKNOWLEDGEMENTS

Ph.D. **Anne Soop**, principal supervisor, for your humanity, encouragement and support. For stepping up as my principal supervisor when I needed one. I am also grateful that you have introduced me to your invaluable network of competent researchers.

Associate professor **Johanna Albert**, co-supervisor, former principal supervisor, for introducing me to research and being an excellent principal supervisor as long as you had the possibility. You are a very wise and have the ability to turn difficult topics into clear matters. Last but not least, you are a rule model and a great travel companion!

Professor **Claes Frostell,** co-supervisor, for your patience and interest in discussing my research (as well as others ☺). Your knowledge about KI management has also been invaluable during my time as a Ph.D. I am also impressed about your ability to never get stressed!

Ph.D. **Lars Hållström**, co-supervisor, for instant answers and your diplomatic skills. Thank you also for intelligent insights when discussing my research, as well as other matters.

Associate professor, **Hans Barle**, my external mentor and also an appreciated colleague. Thank you for providing me about valuable insights about the things surrounding the Ph.D-process that not necessarily involve the very research.

Professor **Håkan Wallén**, vice-head of my KI department, but in front of all an appreciated research partner. Thank you for your optimism, knowledge, and intelligence. You are also a major factor in making my Ph.D. finally come true. I am grateful that you welcomed me into your group of competent researchers and that you hosted us for my final study. In addition to helping me in my research, you seem to have time to encourage every single Ph.D. student of our department, despite many other assignments.

My partners in crime and co-authors Ph.D. **Charlotte Thålin** and Ph.D. **Julie Lasselin**. Without you, **Charlotte** I don´t think I would have made it all the way to dissertation. You have been the "engine" I needed and have engaged yourself in our common research, but also coached me through the writing of this thesis, which is more than was required from you. With you I can share frustration, but in front of all laughters! Thank you!!

Julie, thank you for the splendid collaboration with the human endotoxin study. It is rare to find people you can trust and who really do their part, but you are one of them.

So, **Charlotte and Julie**, without you two my Ph.D. would have become a lot duller. You have acted as my informal mentors and have taught me a lot about research. Not only are you competent researchers but also great friends! Hope to work more with you in the future! In conclusion, this thesis would never have happened without you and your encouragement.

Ph.D. **Fariborz (Fari) Mobarrez** for beeing an excellent researcher as well as contributing to invaluable insights and significant work in paper III.

Professor **Göran Hedenstierna**, for your incomparable excellence and your immense contribution in getting my first paper finally published.

Ph.D. **Annika Lundström**, co-author, for your knowledge about microvesicles and for your brightness and help with my last paper.

Professors **Mats Lekander**, **Mats Ohlsson** and **John Axelsson** (Division of Psychology, Department of Clinical Neuroscience, Karolinska Institutet) for letting me be part of your research and for supporting my own research in many ways.

All staff involved at KFC norr, with a special thanks to nurse **Lena Gabrielsson** and laboratory assistant **Maud Daleskog**. Also, special thanks to nurse **Inga Hellström** for making the intense protocol in the human endotoxin study possible.

My polish research colleagues: **Piotr Harbut**, **Asia Harbut, Stanislaw Zielinski, Waldemar Godzik, Stan Ryniak** and my travel partners/room mates with whom I have been going to Wrosclaw over the years (in addition to my supervisors): **Caroline Haegerstrand, Robert Lindwall, Bartek Jankowski, Johan Lindahl**, **Maria Johnsson and Kristofer Nilsson.** You have made research feel as being on a fun "camp". Ginkoye barzo from Zoska!

The statisticians who have taught me all I know about statistics (and also have had great patience with all my basic questions): **Martin Ålenius**, **Robert Szulkin**, **Fredrik Johansson** and a special thanks to **Jan Kowalski**, my designated " in-case-of emergency" statistician who has also become an appreciated friend.

My "materialare" **Håkan Hansson** och **Johan Lindahl** for invaluable help preparing the human endotoxin study and for being just fabulous guys!

My **colleagues and friends at the Anesthesia and Intensive care department,** Danderyd hospital. Working with you all makes my days (and also nights occasionally ☺).

Lovisa Baer, who apart from being an appreciated colleague and friend, also turned out to be a fabulous artist and ended up painting the front page of this book!

My "roomie" **Tina Linderoth Nagy** for being my debriefing partner at work and for coaching me before all important matters. You are also the most organized person I know, which might be your secret to also being the most skilled scheduler for our department that I have ever seen.

My clinical supervisor **Anette Hein,** for always believing in me. I have really appreciated your endless support and our discussions about career but in front of all life! You have also been my partner in being the endless Ph.D. student. However, I finally won that title graduating 2 months behind you!

Previous and present heads of the Anesthesia and Intensive care department, Danderyd hospital: **Pia Essén**, **Eva Oddby-Muhrbeck**, **Jenny Bång-Arhammar**, for being encouraging and making it possible for me to proceed with my doctoral studies.

My previous and current bosses: **Caroline Haegerstrand** and **Eva Selldén** for supporting my research in many ways. Special thanks to previous boss **Fredrik Hesselvik** who also acted as my co-supervisor during the first half of my thesis.

Head of research in our department, professor **Janne Jakobsson**, aka "the research jet-engine" for making "things" happen and very quickly too! You have also been a great support!

All wonderful secretaries working in the department of Anesthesia and Intensive care, making our administrative life a lot easier. A special thanks to fantastic **Lena Orén**. You are always immensely helpful and happy, despite that I know that you are usually swomped. Another special thanks to **Anna Ridderstrand,** who helped out in the registration of the volunteers for my last study.

My institution **Department of clinical sciences Danderyds hospital**. A special thanks to Professor **Erik Näslund** (head of the department) who always answers instantly no matter what and to **Nina Ringart** for invaluable help with everything that concerns administration (or anything actually!).

My **friends and classmates** from "KIs forskarskola" for all great discussions and a lot of laughters. I am happy we still manage to keep in touch! A special thanks to **Axel Carlsson** and **Ing-Marie Dohrn** for organizing such a great program.

My colleagues from the internship at St Göran Hospital, **Cecilia Engmér, Maria Kugelberg, Andreas Jekell** and **Jonas Brink**. I am so happy that we still keep our tradition with dinner parties in order to keep each other updated about work, but in particular private life.

My fantastic friends. All of you!! A special thanks though to:

Elin and Richard Marsk. **Elin** for all discussions about almost anything (and for listening to my dwelling without complaint!). We have had great fun sharing the interest in music and theatre, even though we don´t always agree with one another (at least not in writing lyrics). However, we always agreed on having "efter-fester" in different shapes. **Richard** for supporting my interest in sports (and being quite patient during my occasional melt downs). I admire your ability of lightening up most things with your amazing sense of humor. It is great to have you in my "sister hood"! You both also have my gratitude for helping me in the final application process for KI research school while I was hospitalized with my son Casper. Love you both!!

Sara Gredmark and **Rusana Simonoska**. I am so happy that we have followed each other since the start of med school. Thank you for all the great conversation and for all your listening over the years. We have shared both happiness and sorrows, but also great travelling experiences. You have also served as my informal external mentors, already being successful researchers.

Malin Vading and **Sofia Sirvell**. You are friends that are always there for me. So happy to have shared so much giggles and travel experiences with you. Thank you for also guiding me through some rougher times of my life.

My parents **Christina Paues Änggård** and **Björn Falkman** for giving me the best possible start in life. I am grateful for all the love and comfort that made me the person I am today. I am also grateful for the endless academic support without any pressure: ”Do what you want, but always do your best”. Thank you also to **Eva Falkman** for the consideration you have provided me over the years and also for making my father happy.

My brothers **Oscar Falkman** and **Göran Paues** for being the persons that your are. **Oscar**, we never shared a home but I have appreciated your company. **Göran**, you were the best person ever to share your childhood with (even if I know I gave you a hard time occasionally over the years). Thank you also for introducing your fantastic wife **Monica** into my life, as well as your two wonderful sons **Isac** and **Alex.**

My aunt **Kari “Tusse” Paues**, for supporting my interest in dogs, even if I disappointed you with my non-existing horse interest. You have coached me through some difficult times in life and I feel that your are always there for me. I am also honored that I am the only child that you have volunteered to baby sit ☺.

The rest of my family, uncles, aunts, cousins (and even further back) both at the **Änggård and Paues** side: Thank you for always taking great interest in my life and for being there.

Gustaf’s family for taking me in. You are all wonderful people and it is an honor for me to be included!

Gustaf, my beloved husband since 11 years and my best friend. Thank you for making me feel special and being the considerate, generous and kind person that you are. Family always comes first for you! Never the least you are also a fantastic father to our children. Love you in so many ways!

Our fantastic children **Theo, Ebba** and **Casper.** You all have so different personalities, but are equally wonderful. Putting you into this world has been my greatest achievement by far. You fill my days with so much joy, laughter, pride and true meaning.

11 REFERENCES

1. Singer, M., et al., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).* JAMA, 2016. **315**(8): p. 801-10.
2. Derek C. Angus, M., MPH, FCCM; Walter T. Linde-Zwirble; Jeffrey Lidicker, MA; Gilles Clermont, MD; Joseph Carcillo, MD; Michael R. Pinsky, MD, FCCM, *Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care.* Crit Care Med 2001. **29**(7): p. 1303-1310.
3. Kumar, G., et al., *Nationwide trends of severe sepsis in the 21st century (2000-2007).* Chest, 2011. **140**(5): p. 1223-31.
4. Stoller, J., et al., *Epidemiology of severe sepsis: 2008-2012.* Journal of Critical Care, 2016. **31**(1): p. 58-62.
5. Friedman, G., J.L. Silva E Fau - Vincent, and J.L. Vincent, *Has the mortality of septic shock changed with time.* Crit Care Med, 1998. **26**(12): p. 2078-86.
6. Angus, D.C. and T. van der Poll, *Severe sepsis and septic shock.* N Engl J Med, 2013. **369**(9): p. 840-51.
7. Greg S. Martin, M.D., David M. Mannino, M.D., Stephanie Eaton, M.D., and Marc Moss, M.D., *The Epidemiology of Sepsis in the United States from 1979 through 2000.* n engl j med 2003. **348**(16): p. 1546-1554.
8. Richard S. Hotchkiss, M.D., and Irene E. Karl, Ph.D., *The pathophysiology and treatment of sepsis.* N Engl J Med. , 2003. **348**(2): p. 138-150.
9. Boomer, J.S., et al., *Immunosuppression in patients who die of sepsis and multiple organ failure.* JAMA, 2011. **306**(23): p. 2594-605.
10. Oberholzer, A., L.L. Oberholzer C Fau - Moldawer, and L.L. Moldawer, *Sepsis syndromes: understanding the role of innate and acquired immunity.* Shock, 2001. **16**(2): p. 83-96.
11. Rittirsch, D., P.A. Flierl Ma Fau - Ward, and P.A. Ward, *Harmful molecular mechanisms in sepsis.* Nat Rev Immunol, 2008. **8**(10): p. 776-87.
12. Remick, D.G., *Cytokine therapeutics for the treatment of sepsis: why has nothing worked?* Curr Pharm Des, 2003. **9**(1): p. 75-82.
13. Bone Rc Fau - Fisher, C.J., Jr., et al., *A controlled clinical trial of high-dose methylprednisolone in the treatment of severe sepsis and septic shock.* N Engl J Med, 1987. **317**(11): p. 653-8.
14. Ziegler, E.J., et al., *Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group.* N Engl J Med, 1991. **324**(7): p. 429-36.
15. van Engelen, T.S.R., et al., *Biomarkers in Sepsis.* Crit Care Clin, 2018. **34**(1): p. 139-152.
16. Biron, B.M., A. Ayala, and J.L. Lomas-Neira, *Biomarkers for Sepsis: What Is and What Might Be?* Biomarker Insights, 2015. **10**(Suppl 4): p. 7-17.

17. Rhodes, A., et al., *Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016.* Crit Care Med, 2017. **45**(3): p. 486-552.

18. Casserly, B., et al., *Lactate measurements in sepsis-induced tissue hypoperfusion: results from the Surviving Sepsis Campaign database.* Crit Care Med, 2015. **43**(3): p. 567-73.

19. Levy, M.M., et al., *2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference.* Intensive Care Med, 2003. **29**(4): p. 530-8.

20. Bone RC, B.R., CerraFB, etal., *American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis.* . Crit Care Med. , 1992. **20**(6): p. 864-874.

21. Vincent, J.L., et al., *The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine.* Intensive Care Med, 1996. **22**(7): p. 707-10.

22. Brink, M., et al., *[New definition of and diagnostic criteria for sepsis - Swedish use of Sepsis-3]. LID - E3W9 [pii].* Lakartidningen, 2018. **115**.

23. Kennedy, M.A., *A brief review of the basics of immunology: the innate and adaptive response.* Vet Clin North Am Small Anim Pract, 2010. **40**(3): p. 369-79.

24. Pancer, Z. and M.D. Cooper, *The evolution of adaptive immunity.* Annu Rev Immunol, 2006. **24**: p. 497-518.

25. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.

26. Yang, H., et al., *High Mobility Group Box Protein 1 (HMGB1): The Prototypical Endogenous Danger Molecule.* Mol Med, 2015. **21** (Suppl 1): p. S6-S12.

27. Bianchi, M.E., *DAMPs, PAMPs and alarmins: all we need to know about danger.* J Leukoc Biol, 2007. **81**(1): p. 1-5.

28. Coll, R.C. and L.A. O'Neill, *New insights into the regulation of signalling by toll-like receptors and nod-like receptors.* J Innate Immun, 2010. **2**(5): p. 406-21.

29. Kawai, T. and S. Akira, *Toll-like receptors and their crosstalk with other innate receptors in infection and immunity.* Immunity, 2011. **34**(5): p. 637-50.

30. Andonegui, G., et al., *Platelets express functional Toll-like receptor-4.* Blood, 2005. **106**(7): p. 2417-23.

31. Stroun, M., et al., *About the possible origin and mechanism of circulating DNA apoptosis and active DNA release.* Clin Chim Acta. **313**(1-2): p. 139-42.

32. Swarup, V. and M.R. Rajeswari, *Circulating (cell-free) nucleic acids--a promising, non-invasive tool for early detection of several human diseases.* FEBS Lett, 2007. **581**(5): p. 795-9.

33. Brinkmann, V., et al., *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**(5663): p. 1532-5.

34. Kenny, E.F., et al., *Diverse stimuli engage different neutrophil extracellular trap pathways.* Elife, 2017. **6**: p. e24437.

35. Papayannopoulos, V. and A. Zychlinsky, *NETs: a new strategy for using old weapons.* Trends Immunol, 2009. **30**(11): p. 513-21.

36. Pilsczek, F.H., et al., *A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus.* J Immunol, 2010. **185**(12): p. 7413-25.

37. Rapala-Kozik, M.Z.a.M., *Neutrophil extracellular traps (NETs) — formation and implications*.* Acta Biochim Pol., 2013. **60**(3): p. 277–284.

38. Byrd, A.S., et al., *An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to Candida albicans.* J Immunol, 2013. **190**(8): p. 4136-48.

39. Mesa, M.A. and G. Vasquez, *NETosis.* Autoimmune Dis, 2013. **2013**(651497).

40. Clark, S.R., et al., *Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood.* Nat Med, 2007. **13**(4): p. 463-9.

41. Tanaka, K., et al., *In vivo characterization of neutrophil extracellular traps in various organs of a murine sepsis model.* PLoS One, 2014. **9**(11): p. e111888.

42. Fattahi, F., et al., *Organ distribution of histones after intravenous infusion of FITC histones or after sepsis.* Immunol Res, 2015. **61**(3): p. 177-86.

43. Logters, T., et al., *The clinical value of neutrophil extracellular traps.* Med Microbiol Immunol, 2009. **198**(4): p. 211-9.

44. Saffarzadeh, M., et al., *Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones.* PLoS One, 2012. **7**(2): p. e32366.

45. Czaikoski, P.G., et al., *Neutrophil Extracellular Traps Induce Organ Damage during Experimental and Clinical Sepsis.* PLoS One, 2016. **11**(2): p. e0148142.

46. Fuchs, T.A., et al., *Extracellular DNA traps promote thrombosis.* Proc Natl Acad Sci U S A, 2010. **107**(36): p. 15880-5.

47. Villanueva, E., et al., *Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus.* J Immunol, 2011. **187**(1): p. 538-52.

48. Narasaraju, T., et al., *Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis.* Am J Pathol, 2011. **179**(1): p. 199-210.

49. Caudrillier, A., et al., *Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury.* J Clin Invest, 2012. **122**(7): p. 2661-71.

50. Kessenbrock, K., et al., *Netting neutrophils in autoimmune small-vessel vasculitis.* Nat Med, 2009. **15**(6): p. 623-5.

51. Wong, S.L., et al., *Diabetes primes neutrophils to undergo NETosis, which impairs wound healing.* Nat Med, 2015. **21**(7): p. 815-9.

52. Young, R.L., et al., *Neutrophil extracellular trap (NET)-mediated killing of Pseudomonas aeruginosa: evidence of acquired resistance within the CF airway, independent of CFTR.* PLoS One, 2011. **6**(9): p. e23637.

53. Demers, M., et al., *Priming of neutrophils toward NETosis promotes tumor growth.* Oncoimmunology, 2016. **5**(5).

54. Cools-Lartigue, J., et al., *Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis.* J Clin Invest, 2013. **123**(8): p. 3446–3458.

55. Park, J., et al., *Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps.* Sci Transl Med, 2016. **8**(361).

56. Demers, M., et al., *Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis.* Proc Natl Acad Sci U S A, 2012. **109**(32): p. 13076-81.

57. Thalin, C., et al., *NETosis promotes cancer-associated arterial microthrombosis presenting as ischemic stroke with troponin elevation.* Thromb Res, 2016. **139**: p. 56-64.

58. Cedervall, J., et al., *Neutrophil Extracellular Traps Accumulate in Peripheral Blood Vessels and Compromise Organ Function in Tumor-Bearing Animals.* Cancer Res, 2015. **75**(13): p. 2653-62.

59. Brinkmann, V., et al., *Neutrophil extracellular traps: how to generate and visualize them.* J Vis Exp, 2010(36).

60. Masuda, S., et al., *NETosis markers: Quest for specific, objective, and quantitative markers.* Clin Chim Acta, 2016. **459**: p. 89-93.

61. van der Vaart M Fau - Pretorius, P.J. and P.J. Pretorius, *The origin of circulating free DNA.* Clin Chem., 2007. **53**(12).

62. Klebanoff, S.J., *Myeloperoxidase: friend and foe.* J Leukoc Biol., 2005. **77**(2): p. 598-625.

63. Korkmaz, B., F. Moreau T Fau - Gauthier, and F. Gauthier, *Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions.* Biochimie, 2008. **90**(2): p. 227-42.

64. Li, Y., et al., *Identification of citrullinated histone H3 as a potential serum protein biomarker in a lethal model of lipopolysaccharide-induced shock.* Surgery, 2011. **150**(3): p. 442-51.

65. Mause, S.F. and C. Weber, *Microparticles: protagonists of a novel communication network for intercellular information exchange.* Circ Res, 2010. **107**(9): p. 1047-57.

66. VanWijk, M.J., *Microparticles in cardiovascular diseases.* Cardiovasc Res, 2003. **59** (2): p. 277–287.

67. Tehrani, S., et al., *Atorvastatin has antithrombotic effects in patients with type 1 diabetes and dyslipidemia.* Thromb Res., 2010. **126**(3): p. e225-31.

68. Sanborn, M.R., et al., *Temporal dynamics of microparticle elevation following subarachnoid hemorrhage.* J Neurosurg, 2012. **117**(3): p. 579-86.

69. Mobarrez, F., et al., *Microparticles and microscopic structures in three fractions of fresh cerebrospinal fluid in schizophrenia: case report of twins.* Schizophr Res, 2013. **143**(1): p. 192-7.

70. Souza, A.C., P.S. Yuen, and R.A. Star, *Microparticles: markers and mediators of sepsis-induced microvascular dysfunction, immunosuppression, and AKI.* Kidney Int, 2015. **87**(6): p. 1100-8.

71. Mastronardi, M.L., et al., *Circulating microparticles from septic shock patients exert differential tissue expression of enzymes related to inflammation and oxidative stress.* Crit Care Med, 2011. **39**(7): p. 1739-48.

72. Delabranche, X., et al., *Microparticles are new biomarkers of septic shock-induced disseminated intravascular coagulopathy.* Intensive Care Med, 2013. **39**(10): p. 1695-703.

73. Zafrani, L., et al., *Calpastatin controls polymicrobial sepsis by limiting procoagulant microparticle release.* Am J Respir Crit Care Med, 2012. **185**(7): p. 744-55.

74. Soop, A., et al., *Effect of lipopolysaccharide administration on the number, phenotype and content of nuclear molecules in blood microparticles of normal human subjects.* Scand J Immunol, 2013. **78**(2): p. 205-13.

75. Mobarrez, F., et al., *The effects of smoking on levels of endothelial progenitor cells and microparticles in the blood of healthy volunteers.* PLoS One, 2014. **9**(2): p. e90314.

76. Ammollo, C.T., et al., *Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation.* J Thromb Haemost, 2011. **9**(9): p. 1795-803.

77. Carestia, A., et al., *Functional responses and molecular mechanisms involved in histone-mediated platelet activation.* Thromb Haemost, 2013. **110**(5): p. 1035-45.

78. Semple, J.W., J.E. Italiano, Jr., and J. Freedman, *Platelets and the immune continuum.* Nat Rev Immunol, 2011. **11**(4): p. 264-74.

79. Iba, T.A.-O.h.o.o. and J.H. Levy, *Inflammation and thrombosis: roles of neutrophils, platelets and endothelial cells and their interactions in thrombus formation during sepsis.* J Thromb Haemost, 2018. **6**(2): p. 231-241.

80. Poli-de-Figueiredo Lf Fau - Garrido, A.G., et al., *Experimental models of sepsis and their clinical relevance.* Shock, 2008. **30 Suppl 1**: p. 53-9.

81. Hawiger, J., *Innate immunity and inflammation: a transcriptional paradigm.* Immunol Res., 2001. **23**(2-3): p. 99-109.

82. Albert, J., et al., *Prolonged exposure to inhaled nitric oxide does not affect haemostasis in piglets.* Intensive Care Med, 2007. **33**(9): p. 1594-601.

83. Da, J.P., L.N. Chen, and G. Hedenstierna, *Nitric oxide up-regulates the glucocorticoid receptor and blunts the inflammatory reaction in porcine endotoxin sepsis.* Critical Care Medicine, 2007. **35**(1): p. 26-32.

84. Suffredini, *Dose-Related Inflammatory Effects of Intravenous Endotoxin in Humans: Evaluation of a New Clinical Lot of Escherichia coli O:113 Endotoxin.* J Infect Dis, 1999: p. 1278-82.

85. Soop, A., et al., *Adenosine treatment attenuates cytokine interleukin-6 responses to endotoxin challenge in healthy volunteers.* Shock, 2003. **19**(6): p. 503-7.

86. Hallstrom, L., et al., *Immunomodulation by a combination of nitric oxide and glucocorticoids in a human endotoxin model.* Acta Anaesthesiol Scand, 2011. **55**(1): p. 20-7.

87. Andreasen, A.S., et al., *Human endotoxemia as a model of systemic inflammation.* Curr Med Chem, 2008. **15**(17): p. 1697-705.

88. Taudorf, S., et al., *Human models of low-grade inflammation: bolus versus continuous infusion of endotoxin.* Clin Vaccine Immunol, 2007. **14**(3): p. 250-5.

89. Berczi I Fau - Bertok, L., T. Bertok L Fau - Bereznai, and T. Bereznai, *Comparative studies on the toxicity of Escherichia coli lipopolysaccharide endotoxin in various animal species.* Can J Microbiol, 1966. **12**(5): p. 1070-1.

90. Slofstra, S.H., H. ten Cate, and C.A. Spek, *Low dose endotoxin priming is accountable for coagulation abnormalities and organ damage observed in the Shwartzman reaction. A comparison between a single-dose endotoxemia model and a double-hit endotoxin-induced Shwartzman reaction.* Thromb J, 2006. **4**: p. 13.

91. Fletcher B, T.F.J., *Stageing of the pathophysiologic responses of the primate microvasculature to Escherichia coli and endotoxin: Examination of the elements of the compensated response and their links to the corresponding uncompensated lethal variants.* Crit Care Med, 2001. **29**((7 Suppl)): p. S78-89.

92. Elinoff, J.M. and A.F. Suffredini, *Exploring the boundaries of systemic inflammation**. Crit Care Med, 2014. **42**(7): p. 1735-7.

93. Deventer, v., *Experimental Endotoxemia in Humans: Analysis of Cytokine Release and Coagulation, Fibrinolytic, and Complement Pathways.* Blood, 1990. **76**(12): p. 2520-2526.

94. Fletcher B, T.F.J., *Two-stage response to endotoxin infusion into normal human subjects: Correlation of blood phagocyte luminescence with clinical and laboratory markers of the inflammatory, hemostatic response.* . Crit Care Med 2001. **29**: p. 326 – 334.

95. Suffredini, *Promotion and subsequent inhibition of plasminogen activation after administration of intravenous endotoxinto normal subjects.* N Engl J Med, 1989. **320**(18): p. 1165-72.

96. Silverstein, R., et al., *Differential host inflammatory responses to viable versus antibiotic-killed bacteria in experimental microbial sepsis.* Infect Immun, 2000. **68**(4): p. 2301-8.

97. Dejager, L., et al., *Cecal ligation and puncture: the gold standard model for polymicrobial sepsis?* Trends Microbiol, 2011. **19**(4): p. 198-208.

98. Frostell, C., *Inhaled Nitric Oxide. A Selective Pulmonary Vasodilator Reversing Hypoxic Pulmonary Vasoconstriction.* Circulation, 1991. **83**(6): p. 2038-47.

99. Rossaint R1, P.U., Gerlach H, Falke KJ., *Inhaled nitric oxide: its effects on pulmonary circulation and airway smooth muscle cells.* Eur Heart J., 1993. **14**(Suppl I): p. 133-40.

100. Mali Mathru, M.D.R.H., Ph.D.; Daneshvari R. Solanki, F.R.C.A.; Stephen Hays, R.R.T.; John D. Lang, M.D., *Inhaled Nitric Oxide Attenuates Reperfusion Inflammatory Responses in Humans.* Anesthesiology 2007. **106**(3): p. 275-282.

101. Gozdzik, W., et al., *Prolonged exposure to inhaled nitric oxide transiently modifies tubular function in healthy piglets and promotes tubular apoptosis.* Acta Physiol (Oxf), 2009. **195**(4): p. 495-502.

102. Weitzberg , H.M., Lundberg JO, *Nitrate-Nitrite-Nitric Oxide Pathway: Implications for Anesthesiology and Intensive Care.* Anesthesiology, 2010. **113**(6): p. 1460-75.

103. Bogdan , R.M., Diefenbach A, *The role of nitric oxide in innate immunity.* Immunological_Reviews., 2000. **173**: p. 17-26.

104. Kinsella, J.P., et al., *Effects of inhaled nitric oxide on pulmonary edema and lung neutrophil accumulation in severe experimental hyaline membrane disease.* Pediatr Res, 1997. **41**(4 Pt 1): p. 457-63.

105. Kang JL, P.W., Pack IS, Lee HS, Kim MJ, Lim CM, Koh Y., *Inhaled nitric oxide attenuates acute lung injury via inhibition of nuclear factor-kappa B and inflammation.* J Appl Physiol 2002. **92**(2): p. 795-801.

106. Phillips, L., et al., *Nitric oxide mechanism of protection in ischemia and reperfusion injury.* J Invest Surg, 2009. **22**(1): p. 46-55.

107. Chadda K, A.D., *The use of corticosteroids in severe sepsis and acute respiratory distress syndrome.* Ann Med. , 2002. **34**(7-8): p. 582-9.

108. Annane, D., et al., *Impaired pressor sensitivity to noradrenaline in septic shock patients with and without impaired adrenal function reserve.* Br J Clin Pharmacol., 1998. **46**(6): p. 589-97.

109. Djillali Annane, M., et al., *Corticosteroids in the Treatment of Severe Sepsis and Septic Shock in Adults. A Systematic Review.* JAMA, 2009. **301**(22): p. 2362-2375.

110. Sligl, W.I., et al., *Safety and efficacy of corticosteroids for the treatment of septic shock: A systematic review and meta-analysis.* Clin Infect Dis, 2009. **49**(1): p. 93-101.

111. Venkatesh, B., et al., *Adjunctive Glucocorticoid Therapy in Patients with Septic Shock.* . N Engl J Med, 2018. **378**(9): p. 797-808.

112. Sprung, C.L., et al., *Hydrocortisone therapy for patients with septic shock.* N Engl J Med, 2008. **358**(2): p. 111-24.

113. Li, P., et al., *Regulation of p53 target gene expression by peptidylarginine deiminase 4.* Mol Cell Biol., 2008. **28**(15): p. 4745-58.

114. *FDA Guidance for Industry: Bioanalytical Method Validation.* 2001.

115. McCoy, J.P., Jr., *Basic principles of flow cytometry.* Hematol Oncol Clin North Am, 2002. **16**(2): p. 229-43.

116. Ma, A.C. and P. Kubes, *Platelets, neutrophils, and neutrophil extracellular traps (NETs) in sepsis.* J Thromb Haemost, 2008. **6**(3): p. 6(3):415-20.

117. Thalin, C.A.-O., et al., *Citrullinated histone H3 as a novel prognostic blood marker in patients with advanced cancer.* PLoS One, 2018. **13**(1): p. e0191231.

118. Mauracher, L.M., et al., *Citrullinated histone H3, a biomarker of neutrophil extracellular trap formation, predicts the risk of venous thromboembolism in cancer patients.* J Thromb Haemost, 2018. **16**(3): p. 508-518.

119. Ekaney Ml Fau - Otto, G.P., et al., *Impact of plasma histones in human sepsis and their contribution to cellular injury and inflammation.* Crit Care, 2014. **18**(5): p. 543.

120. Wang, Y., et al., *Neutrophil extracellular trap-microparticle complexes enhance thrombin generation via the intrinsic pathway of coagulation in mice.* Sci Rep, 2018. **8**(1): p. 4020.

121. Gavillet, M., et al., *Flow cytometric assay for direct quantification of neutrophil extracellular traps in blood samples.* Am J Hematol, 2015. **90**(12): p. 1155-8.

122. Lee, K.H., et al., *Quantification of NETs-associated markers by flow cytometry and serum assays in patients with thrombosis and sepsis.* Int J Lab Hematol, 2018.

123. Manda-Handzlik, A., et al., *Flow cytometric quantification of neutrophil extracellular traps: Limitations of the methodological approach.* Am J Hematol. **91**(3): p. E9-10.

124. Zhao, W., D.K. Fogg, and M.J. Kaplan, *A novel image-based quantitative method for the characterization of NETosis.* J Immunol Methods, 2015. **423**: p. 104-10.

125. Haase, N., et al., *Hydroxyethyl starch 130/0.38-0.45 versus crystalloid or albumin in patients with sepsis: systematic review with meta-analysis and trial sequential analysis.* BMJ, 2013. **346**(f839).

126. Gozdzik, W., et al., *Beneficial effects of inhaled nitric oxide with intravenous steroid in an ischemia-reperfusion model involving aortic clamping.* Int J Immunopathol Pharmacol, 2018. **32**.

127. Troncy, E., et al., *Extra-pulmonary effects of inhaled nitric oxide in swine with and without phenylephrine.* Br J Anaesth, 1997. **79**(5): p. 631-40.

128. Gebistorf, F., et al., *Inhaled nitric oxide for acute respiratory distress syndrome (ARDS) in children and adults.* Cochrane Database Syst Rev, 2016(6): p. Cd002787.

129. Ranieri Vm Fau - Rubenfeld, G.D., et al., *Acute respiratory distress syndrome: the Berlin Definition.* JAMA, 2012. **307**(23): p. 2526-33.

130. Krafft P1, F.P., Fitzgerald RD, Koc D, Steltzer H., *Effectiveness of Nitric Oxide Inhalation in Septic ARDS.* Chest, 1996. **109**(2): p. 486-93.

131. Gerlach, H., et al., *Time-course and dose-response of nitric oxide inhalation for systemic oxygenation and pulmonary hypertension in patients with adult respiratory distress syndrome.* Eur J Clin Invest, 1993. **23**(8): p. 499-502.

132. Cannon, R.O., 3rd, et al., *Effects of inhaled nitric oxide on regional blood flow are consistent with intravascular nitric oxide delivery.* J Clin Invest, 2001. **108**(2): p. 279-87.

133. Ng, E.S., et al., *Enhanced S-nitroso-albumin formation from inhaled NO during ischemia/reperfusion.* Circ Res, 2004. **94**(4): p. 559-65.

134. Dellinger, R.P., et al., *Effects of inhaled nitric oxide in patients with acute respiratory distress syndrome: results of a randomized phase II trial. Inhaled Nitric Oxide in ARDS Study Group.* Crit Care Med, 1998. **26**(1): p. 15-23.

135. Sun, Q., et al., *Optimal adrenergic support in septic shock due to peritonitis.* Anesthesiology, 2003. **98**(4): p. 888-96.

136. Steinberg, J., et al., *The development of acute respiratory distress syndrome after gut ischemia/reperfusion injury followed by fecal peritonitis in pigs: a clinically relevant model.* Shock, 2005. **23**(2): p. 129-37.

137. Kieslichova, E., et al., *The effect of immunosuppression on manifestations of sepsis in an animal model of cecal ligation and puncture.* Transplant Proc, 2013. **5**(2): p. 770-7.

138. Soerensen, K.E., et al., *The use of sequential organ failure assessment parameters in an awake porcine model of severe Staphylococcus aureus sepsis.* APMIS, 2012. **120**(11): p. 909-21.

139. Ripanti, D., et al., *Application of the Sequential Organ Failure Assessment Score to predict outcome in critically ill dogs: preliminary results.* Schweiz Arch Tierheilkd, 2012. **154**(8): p. 325-30.

140. McConachie, E., S. Giguere, and M.H. Barton, *Scoring System for Multiple Organ Dysfunction in Adult Horses with Acute Surgical Gastrointestinal Disease.* J Vet Intern Med, 2016. **30**(4): p. 1276-83.

141. Martinod, K. and D.D. Wagner, *Thrombosis: tangled up in NETs.* Blood, 2014. **123**(18): p. 2768-76.

142. Martinod, K., et al., *Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice.* Proc Natl Acad Sci U S A, 2013. **110**(21): p. 8674-9.

143. Gould, T.J., et al., *Cell-Free DNA Modulates Clot Structure and Impairs Fibrinolysis in Sepsis.* Arterioscler Thromb Vasc Biol, 2015. **35**(12): p. 2544-53.

144. Xu, J., et al., *Extracellular histones are major mediators of death in sepsis.* Nat Med, 2009. **15**(11): p. 1318-21.

145. Keshari, R.S., et al., *Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition.* PLoS One., 2012. **7**(10): p. e48111.

146. Yamashita, H., et al., *Inhaled nitric oxide attenuates apoptosis in ischemia-reperfusion injury of the rabbit lung.* Ann Thorac Surg, 2004. **78**(1): p. 292-7.

147. Zhang, J., et al., *Administration of dexamethasone protects mice against ischemia/reperfusion induced renal injury by suppressing PI3K/AKT signaling.* Int J Clin Exp Pathol, 2013. **6**(11): p. 2366-75.

148. Biron, B.M., et al., *Cl-Amidine Prevents Histone 3 Citrullination and Neutrophil Extracellular Trap Formation, and Improves Survival in a Murine Sepsis Model.* J Innate Immun, 2017. **9**(1): p. 22-32.

149. Pan, B., et al., *CitH3: a reliable blood biomarker for diagnosis and treatment of endotoxic shock.* Sci Rep, 2017. **7**(1): p. 8972.

150. Yang, S., et al., *Neutrophil Extracellular Traps Promote Hypercoagulability in Patients With Sepsis.* Shock, 2017. **47**(2): p. 132-139.

151. McDonald, B., et al., *Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice.* Blood, 2017. **129**(10): p. 1357-1367.

152. Li, Y., et al., *Citrullinated histone H3: a novel target for the treatment of sepsis.* Surgery, 2014. **156**(2): p. 229-34.

153. Meng W Fau - Paunel-Gorgulu, A., et al., *Depletion of neutrophil extracellular traps in vivo results in hypersusceptibility to polymicrobial sepsis in mice.* Crit Care, 2012. **16**(4).

154. Biron, B.M., et al., *PAD4 Deficiency Leads to Decreased Organ Dysfunction and Improved Survival in a Dual Insult Model of Hemorrhagic Shock and Sepsis.* J Immunol, 2018. **200**(5): p. 1817-1828.

155. Hemmers, S., et al., *PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection.* PLoS One, 2011. **6**(7): p. e22043.

156. Martinod, K., et al., *PAD4-deficiency does not affect bacteremia in polymicrobial sepsis and ameliorates endotoxemic shock.* Blood, 2015. **125**(12): p. 1948-56.

157. Hallstrom, L., et al., *No signs of inflammation during knee surgery with ischemia: a study involving inhaled nitric oxide.* Mediators Inflamm, 2014. **2014**: p. 620281.

158. Lang, J.D., Jr., et al., *Inhaled NO accelerates restoration of liver function in adults following orthotopic liver transplantation.* J Clin Invest, 2007. **117**(9): p. 2583-91.

159. Liu, X., et al., *Nitric oxide inhalation improves microvascular flow and decreases infarction size after myocardial ischemia and reperfusion.* J Am Coll Cardiol, 2007. **50**(8): p. 808-17.

160. Kim, M.D.P.D.Yong L., M.D.Kenneth A. Richman, and M.D.F.R.C.P.Bryan E. Marshall, *Thrombocytopenia Associated with Swan-Ganz Catheterization in Patients.* Anesthesiology, 1980. **53**(3): p. 261-262.

161. Rhodes, A., et al., *A randomised, controlled trial of the pulmonary artery catheter in critically ill patients.* Intensive Care Med, 2002. **28**(3): p. 256-64.

162. Vicente Rull Jr Fau - Loza Aguirre, J., et al., *Thrombocytopenia induced by pulmonary artery flotation catheters. A prospective study.* Intensive Care Med, 1984. **10**(1): p. 29-31.

163. Fuchs, T.A., D.D. Brill A Fau - Wagner, and D.D. Wagner, *Neutrophil extracellular trap (NET) impact on deep vein thrombosis.* Arterioscler Thromb Vasc Biol, 2012. **32**(8): p. 1777-83.

164. Hampson, P., et al., *Neutrophil Dysfunction, Immature Granulocytes, and Cell-free DNA are Early Biomarkers of Sepsis in Burn-injured Patients: A Prospective Observational Cohort Study.* Ann Surg, 2016. **265**(6): p. 1241-1249.

165. Lashin, H.M.S., et al., *Microvesicle Subsets in Sepsis Due to Community Acquired Pneumonia Compared to Faecal Peritonitis.* Shock, 2018. **49**(4): p. 393-401.

166. Margraf, S., et al., *Neutrophil-derived circulating free DNA (cf-DNA/NETs): a potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis.* Shock, 2008. **30**(4): p. 352-8.

167. Kaufman, T., et al., *Nucleosomes and neutrophil extracellular traps in septic and burn patients.* Clin Immunol, 2017. **183**: p. 254-262.

168. Delabranche, X., et al., *Evidence of Netosis in Septic Shock-Induced Disseminated Intravascular Coagulation.* Shock, 2016. **47**(3): p. 313-317.