The interplay between stress hormones, mitochondria, and immune cells during sepsis; from adaptation to maladaptation

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I, Miranda Jentine Melis, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Background Sepsis is a severe dysregulated response of the body to infection, leading to multiple organ dysfunction. This phenomenon, and immunosuppression seen with prolonged sepsis, may relate to insufficient mitochondrial production of ATP. The magnitude of endocrine changes during sepsis has major prognostic implications. These could significantly contribute to decreased mitochondrial activity, and in turn, functional disturbances of immune cells.

Objectives To study the effects of changes in stress and metabolic hormones during sepsis on mononuclear immune cell mitochondrial and effector function.

Methods Endocrine, mitochondrial, and inflammatory markers were measured in serum and monocytes taken from septic patients, with subsequent correlations calculated between these markers. Direct effects of a selection of hormones (noradrenaline, adrenaline, and hydrocortisone) on mitochondrial and mononuclear immune cell function were examined in a 6-hour *in vitro* model of infection. A rat model of faecal peritonitis was then characterised for endocrine, metabolic, and inflammatory parameters for future *in vivo* studies.

Results There were no consistent correlations between endocrine levels and various measures of mitochondrial and mononuclear immune cell function in patients with sepsis on admission nor in pre-operative control patients. Mitochondrial dysfunction could not explain changes in immune cell effector function both in patients nor *in vitro*. *In vitro* studies indicate that catecholamine- and glucocorticoid-induced effects on mitochondrial and immune function are dose, stimulation, and cell-type specific.

Conclusion Opposite directions of correlations between endocrine levels and mitochondrial and mononuclear immune cell function suggest a change in regulation of these processes. The variable *in vitro* effects in terms of dose, stimulation and cell

type highlight the importance of stratifying patients to identify who could benefit from manipulation of hormone levels. Further observational, *in vitro* and *in vivo* studies are warranted to confirm and elaborate on these observations made, and to study potential interventions to restore mitochondrial and immune cell effector function.

Impact statement

Sepsis is a major health problem worldwide, with high mortality and morbidity rates. Besides being a threat to human health, it imposes a major economic burden on healthcare systems and affected individuals. Despite this, the exact pathophysiology of sepsis has not yet been elucidated and only limited supportive treatment options are currently available. The accumulated failures from translating preclinical research outcomes could be explained by the use of non-representative animal models and by the heterogeneity of sepsis. This makes this syndrome a highly complex field of study. No single intervention will work in all patients, making it a challenge to distinguish those who will benefit from those who will not, and especially from those who might be harmed.

My host laboratory has a particular interest in the role of mitochondrial dysfunction in sepsis, ultimately affecting organ function, including cells of the immune system. Failed organs in patients appear largely normal histologically, suggesting a functional rather than structural abnormality. Decreased mitochondrial activity and a metabolic shutdown could well explain this phenomenon. This state of metabolic dormancy could be induced via hormone-mediated effects leading to immunosuppression. Changes in stress and metabolic hormones likely play a crucial but underappreciated role in this process. Some of these hormones are administered exogenously as part of sepsis therapy, in addition to being released endogenously.

To study the above phenomenon, I utilised three different models. These include an observational study with septic patients, an *in vitro* model of infection with primary human immune cells, and an animal model of faecal peritonitis. Although my results are of interest, at this stage, it is too early to make any strong recommendations on a clinical practice level. My results have however laid useful groundwork for further exploration and have been shared in various presentations at national and

international conferences. In the short term, these findings will allow colleagues to further unpick the underlying mechanisms of stress and metabolic hormones affecting mitochondrial and immune cell function, and how to subsequently manipulate these. In the longer term, these findings could influence clinical practice and benefit patients suffering under these conditions.

An elaborate characterisation of the temporal stress response in the lab's animal model of faecal peritonitis – including metabolic, endocrine, and inflammatory markers – has been compared to that in patients. These results are currently being written up for publication. With current concern about the fidelity of rodent models of sepsis, this will provide the scientific community with extensive knowledge on the differences and similarities between the two. This information is important to studies of physiology, diagnostics, and interventions in sepsis, and will hopefully result in better selection of animal models, ultimately preventing unnecessary animal use in science, and improving translatability of outcomes and patient safety.

Besides the findings presented in this thesis, various laboratory protocols have been set up. These include rat hepatocyte isolations, respirometry of hepatocytes and immune cells, and measurement of mitochondrial function of cells by flow cytometry. Passing on the principles of these techniques to colleagues, there is potential for future utilisation of these locally. In addition, patient samples that were collected as part of this thesis are currently being used by other members of the lab studying thermogenic mechanisms in sepsis. This serum can be used to induce an *in vitro* septic milieu. Samples collected from our rat model could be used for a similar purpose. This optimised sample and data utilisation prevents unnecessary exposure of patients to observational studies and sample collection, and the use of animals in research.

Acknowledgements

What a journey it has been. I cannot believe it has already been almost 5 years since I arrived in London to start my PhD in the famous Singer lab. It was a gamble, not having seen the lab in person before I decided to move from Melbourne to London. Unsurprisingly for any PhD student, I have experienced many ups and downs during my time here, with lab closures due to the corona pandemic being one of them. That being said, I also found my next destination in life, and have met some incredible people along the way. This is my way of saying thank you to all those wonderful colleagues, friends, and family who have supported me throughout this journey.

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ADl/m/h	Adrenaline low/medium/high
AR	Adrenergic Receptor
ACTH	Adrenocorticotropic Hormone
ANF	Atrial Natriuretic Factor
ANOVA	Analysis of Variance
ANT	Adenosine Nucleotide Transferase
AP-1	Activator Protein-1
APACHE II	Acute Physiology and Chronic Health Evaluation II
APC	Antigen Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine Triphosphate
A.U.	Arbitrary Units
AUROC	Area Under the ROC
BAT	Brown Adipose Tissue
Bcl-2	B-cell Lymphoma 2
BCR	B-Cell Receptor
BCT	Blood Collection Tube
BGA	Blood Gas Analysis
BNP	B-type Natriuretic Peptide
BP	Blood Pressure
BSU	Biological Services Unit
cAMP	Cyclic Adenosine Monophosphate
C	Cytochrome C
CCI	Charlson's Comorbidity Index
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CI	Confidence Interval
CLAMS	Comprehensive Lab Animal Monitoring System

CO	Cardiac Output
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Coronavirus Disease 2019
CoQ	Coenzyme Q10
CREB	cAMP-Response Element Binding Protein
CRH	Corticotropin-Releasing Hormone
CS	Citrate Synthase
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein-4
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DEHA	Dehydroepiandrosterone
dH_2O	De-ionised H_2O
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DO_2	O_2 Delivery
EC	*Escherichia Coli*
ECAR	Extracellular Acidification Rate
ECR	Early Career Researcher
ELISA	Enzyme-Linked Immunosorbent Assays
ER	Endoplasmic Reticulum
ERK1/2	Extracellular Signal-Regulated Kinase
ESA	European Sepsis Academy
ETC	Electron Transport Chain
EU	European Union
f	Female
$FAD(H_2)$	Flavin Adenine Dinucleotide
FBS	Fetal Bovine Serum
FCCP	Carbonyl Cyanide-P-(Trifluoromethoxy) Phenylhydrazone
FcR	Fc Receptor
FiO_2	Fractional Inspired O_2
FMO	Fluorescence Minus One

FOXO1	Forkhead Box O1
FSC	Forward Scatter
fT_3	Free T_3
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GSH	Glutathione
h	Hour
Hb	Haemoglobin
HBSS	Hank's Balances Salt Solution
HCl/m/h	Hydrocortisone low/medium/high
HCO_3^-	Bicarbonate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HIV	Human Immunodeficiency Virus
HKB	Heat Killed Bacteria
HLA	Human Leukocyte Antigen
HPA	Hypothalamus-Pituitary-Adrenal
HPT	Hypothalamus-Pituitary-Thyroid
HR	Heart Rate
HSL	Hormone-Sensitive Lipase
ICF	Informed Consent Form
ICU	Intensive Care Unit
Ig	Immunoglobulin
IGF	Insulin Growth Factor
IL	Interleukin
IMM	Inner Mitochondrial Membrane
INF	Interferon
i.p.	Intraperitoneal
IP	IFN-y Inducible Protein
IP_3	Inositol Triphosphate
IQR	Interquartile Range
IRF-3	INF Response Factor-3

IRS	Insulin Receptor Substrate
i.v.	Intravenous
L-DOPA	L-3,4-Dihydroxyphenylalanine
LPS	Lipopolysaccharide
LSD	Least Significant Difference
m	Male
MAP	Mean Arterial Pressure
MAPK	Mitogen-Activated Protein Kinase
MCP	Methylcyclopropene
MDSC	Myeloid-Derived Suppressor Cells
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIF	Macrophage-Inhibiting Factor
MMP	Mitochondrial Membrane Potential
MnSOD	Manganese Superoxide Dismutase
MOF	Multiple Organ Failure
mPTP	Mitochondrial Permeability Transition Pore
MQTiPSS	Minimum Quality Threshold in Pre-Clinical Sepsis Studies
MR	Mineralocorticoid Receptor
mROS	Mitochondrial ROS
MS	Multiple Sclerosis
mtDNA	Mitochondrial DNA
mTOR	Mammalian Target of Rapamycin
mtRNA	Mitochondrial RNA
MyD88	Myeloid Differentiation Primary Response 88
NAD(H)	Nicotinamide Adenine Dinucleotide
NADPH	NADH Phosphate
NAl/m/h	Noradrenaline low/medium/high
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear Factor Kappa B
NK	Natural Killer

NLRP3	NLR family Pyrin domain containing 3
NO	Nitric Oxide
NOX2	NADPH Oxidase 2
NR	Normal Range
NRF	Nuclear Respiratory Factor
NTI	Non-Thyroidal Illness
O_2^-	Superoxide
OD	Optical Density
OXPHOS	Oxidative Phosphorylation
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
pCO_2	Partial Pressure of CO_2
PD-1	Programmed Cell Death-1
PGC-1α	PPAR-γ Coactivator 1α
PKA	Protein Kinase A
PMA	Phorbol Myristol Acetate
pO_2	Partial Pressure of O_2
PRR	Pattern Recognition Receptors
PVC	Polyvinyl Chloride
P13k	Phosphoinositide 3-Kinase
P2Y2R	P2Y2 Receptor
P38	Protein 38
RAGE	Receptor for Advanced Glycation Endproducts
RCR	Respiratory Control Ratio
RER	Respiratory Exchange Ratio
rT_3	Reverse T_3
RNS	Reactive Nitrogen Species
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute

SA	*Staphylococcus Aureus*
s.c.	Subcutaneous
SD	Standard Deviation
SIRT	Sirtuins
SLE	Systemic Lupus Erythematosus
SNS	Sepsis Non-Survivor
SOFA	Sequential Organ Failure Assessment
sO_2	O_2 Saturation
SS	Sepsis Survivor
SSC	Sideward Scatter
STRESS	Studying Responses of the Stress System
SV	Stroke Volume
T_3	Triiodothyronine
T_4	Thyroxine
TB	Tuberculosis
TBG	Thyroxine-Binding Globulin
TCA	Tricarboxylic Acid Cycle
TCR	T-Cell Receptor
Tfam	Transcription Factor A for the Mitochondrion
TGF	Transforming Growth Factor
Th	T-Helper
TLR	Toll-Like Receptor
TMRM	Tetramethyl-Rhodamine Methyl Ester
TNF	Tumor Necrosis Factor
TR	Thyroid hormone Receptor
TRE	Thyroid Response Element
Treg	Regulatory T-Cell
TRH	Thyroid-Releasing Hormone
TSH	Thyroid-Stimulating Hormone
TTE	Transthoracic Echocardiography
TTR	Transthyretin

UCL	University College London
UCLH	UCL Hospital
UCP	Uncoupling Protein
US	Unstimulated
VCO_2	CO_2 Production
VO_2	O_2 Consumption
VTI	Velocity-Time Integral
WAT	White Adipose Tissue
WBC	White Blood Count
WHO	World Health Organisation

Chapter 1 Introduction

1.1 Sepsis definition and epidemiology

Sepsis is a syndrome defined as a life-threatening dysregulated response of the body to infection, leading to Multiple Organ Failure (MOF) [1]. This latest definition emphasises the role of the host response, rather than the triggering infection, in driving the pathogenesis of sepsis. Septic shock is a subset of sepsis with profound circulatory, cellular, and metabolic abnormalities, and is associated with a greater risk of mortality than sepsis alone [2]. It is a significant cause of morbidity and mortality worldwide, with incidences varying by geographical location and patient characteristics such as age, comorbidities, and the number and types of organ dysfunction [2, 3]. Because of its high global burden, sepsis has been recognised by the World Health Organisation (WHO) as a global health priority [4].

In some reports, rates of sepsis incidence are increasing, though these are largely built around extrapolated data, with very limited data from low and middle-income countries [4]. One study suggested 31.5 million sepsis cases annually and 5 million deaths [5], while another estimated 48.9 million sepsis cases worldwide in 2017, and 11 million sepsis-related deaths, representing 19.7% of all global deaths [6]. Nonetheless, according to the model used in this study, sepsis incidence fell by 37%, and mortality decreased by 52.8% compared to 1990. Variability in case definition and different methods of care registration also hinder precise documentation and estimation of sepsis incidence and mortality [7].

In developed countries, most hospitalised cases of sepsis with significant organ dysfunction are managed in the Intensive Care Unit (ICU), unless the patient's underlying frailty or severe comorbidities (e.g. terminal cancer) dictate that aggressive intensive care life support would be futile and not in their best interest. Sepsis

management remains largely supportive, with eradication of the infection with antibiotics and source control, and organ support such as fluid and vasoactive drug administration, mechanical ventilation, and renal replacement therapy [8]. No specific treatment directed at the host response has been shown to be of benefit, despite multiple large-scale clinical trials performed over the last few decades [9].

Survivors often suffer from a variety of long-term effects, including cognitive and physical impairment, and cardiovascular disease [10, 11]. In addition, patients commonly experience re-hospitalisation, inability to resume prior employment, persistent immunosuppression making them vulnerable to secondary infection, and reduced quality of life [12]. The high incidence of sepsis and these frequent, long-term consequences make it a significant financial burden to society [13].

1.2 Sepsis pathophysiology

The host response to sepsis consists of simultaneous activation or suppression of multiple pathways. Sepsis starts with the recognition of a pathogen by the immune system, leading to a systemic inflammatory response and subsequent activation of a range of complex and biphasic interactions (Figure 1). These interactions involve autonomic, cardiovascular, endocrine, metabolic, bioenergetic and immune systems, and multiple signalling pathways within these systems. The pathways involved are interlinked, but precise interactions remain unclear. These reactions ultimately result in MOF requiring drug and device support [14]. Changes in immune cell regulation, hormones, metabolism, and mitochondria will be discussed in more detail.

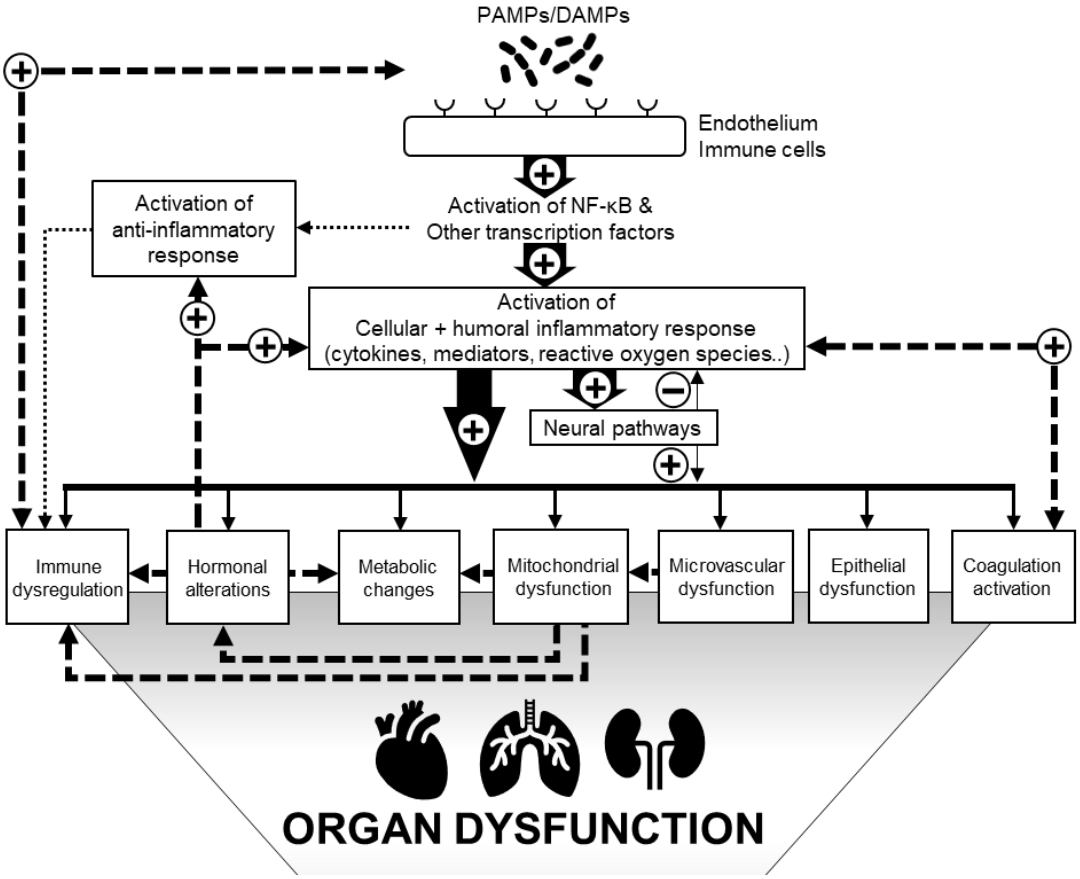

Figure 1. Interactions between signalling pathways involved in sepsis pathology leading to organ dysfunction. The recognition of a pathogen by the immune system leads to activation of transcription factors, an inflammatory response, and activation of a range of complex and biphasic interactions DAMP: Damage-Associated Molecular Pattern; NF-kB: Nuclear Factor Kappa B; PAMP: Pathogen-Associated Molecular Pattern. Adapted from [14].

1.2.1 Immune (dys)regulation

The inflammatory host response to sepsis is initiated when Pattern Recognition Receptors (PRRs) on immune and endothelial cells encounter the presence of microbial products. These include Pathogen-Associated Molecular Patterns (PAMPs) released from the infecting microorganism, as well as Damage-Associated Molecular Patterns (DAMPs), such as Mitochondrial Deoxyribonucleic Acid (mtDNA), heat shock proteins, and histones, which are released from their intracellular compartment following injury [15].

The subsequent host response is characterised by a cascade of inflammatory mediators that trigger downstream processes that aim to defend the body, but may paradoxically make it more vulnerable. This response involves excessive inflammation, immune suppression, and a failure to return to normal homeostasis. Treatment strategies for sepsis have been largely aimed at suppressing excessive hyper-inflammation with anti-inflammatory strategies but, in recent years, there has been increasing interest in activation of the suppressed immune system [16, 17]. Excellent reviews have been published on the immune response during sepsis [17-19]; a brief overview of immune function in health and sepsis is given below.

1.2.1.1 The immune system

The immune system is comprised of a plethora of barriers, cells, and effector molecules. When pathogens pass the first line defence system (epithelium, mucus membranes), they encounter cells of the innate immune system. The innate immune response is rapid, non-specific, and consists of cellular and non-cellular physical and chemical elements. Innate cells recognise PAMPs and DAMPs using distinct PRRs expressed on their surface. These distinct classes of PRRs recognise different pathogens or products [20]. Activation of PRRs transduces signals into an inflammatory response via activation of inflammasome complexes or by induction and translocation of nuclear factor-κ B (NF-κB) into the nucleus and subsequent target gene activation [17, 21]. These trigger a downstream inflammatory-immune response that walls off the infection to prevent its spread, attracts other immune cells (via chemokines), and activates these cells (via cytokines) to control the infection [15].

Although the innate immune system was originally thought to have no memory function, studies have introduced the concept of trained immunity [22]. The innate immune system consists of cells of the myeloid lineages, including monocytes, macrophages, neutrophils, Dendritic Cells (DCs), and Natural Killer (NK) cells. The

first three types are phagocytic cells that can engulf and kill pathogens. Monocytes, macrophages and DCs are Antigen-Presenting Cells (APCs) that trigger the adaptive immune response by presenting antigens, using Major Histocompatibility Complexes (MHCs) for recognition. NK cells help mobilise APCs via secretion of Interferon (INF)-γ and release of perforins and granzymes that induce apoptosis [23].

Considering the topic of this thesis, extra light will be shed upon monocytes. These bone marrow-derived cells represent 5-10% of circulating leukocytes and persist in the periphery for several days [24]. They can ultimately differentiate into macrophages or DCs. Effector functions differ slightly for monocyte subtypes that are distinguished by Cluster of Differentiation (CD)14 and CD16 surface expression. The monocytes can be divided into classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$) and non-classical ($CD14^{+}/CD16^{++}$) [25]. Most monocytes are classical and have a predominantly phagocytic phenotype [26]. Non-classical monocytes have an increased ability to produce pro-inflammatory cytokines and present antigens, while intermediate monocytes form a small proportion of blood monocytes and have a transitional phenotype [27].

In addition to the innate immune cells described above, the innate system also includes mast cells, basophils, eosinophils, innate lymphoid cells, and various non-cellular elements such as complement system proteins produced by the liver [23]. These proteins act as pro-inflammatory molecules to recruit phagocytic cells and induce a cascade that opsonises pathogens for phagocytosis by other cells. There are three pathways that induce complement activation: i) classical, ii) mannose-binding lectin, and iii) alternative [28, 29]. Other non-cellular elements include acute-phase proteins, anti-microbial peptides, secretory Immunoglobulin (Ig)A and circulatory IgM [30].

When the innate immune system becomes ineffective in eliminating pathogens, the interplay between innate and adaptive immune systems becomes crucial. The

adaptive immune system has memory capacity, making it a rapid and efficient response upon secondary exposure to a similar antigen. Although antigen-dependent and specific, response times are longer compared to the innate immune system [23].

Cells of the adaptive immune system include B-cells and T-cells. B-cells arise from haematopoietic stem cells in the bone marrow where they mature. These cells can directly recognise antigens with specific B-cell Receptors (BCRs) without involvement of other APCs. They even function as APCs themselves by presenting antigens to $CD4^+$ T-cells, using MHC II surface molecules. B-cells proliferate and differentiate into antibody-secreting plasma cells or memory B-cells. T-Helper (Th) cells help B-cells to differentiate into these plasma cells to produce various antibodies (e.g. IgA, IgD, IgE, IgG, and IgM) which can bind pathogens for neutralisation, complement activation, and opsonisation. B-cells are generally long-lived and respond quickly upon antigen exposure [23].

Like B-cells, T-cells are derived from the bone marrow but mature in the thymus. They express antigen-binding receptors known as T-Cell Receptors (TCRs) that can be activated by antigens on MHC surface molecules on APCs. Upon binding, T-cells proliferate, secrete cytokines, and differentiate into various subtypes. These subtypes consist of $CD8^+$ cytotoxic T-cells that destroy infected cells and are important in the elimination of tumour cells, $CD4^+$ Th cells (Th1, Th2 and Th17 being most frequent) that are potent cytokine secretors, and Regulatory $CD4^+$ T-cells (Treg), a subset that suppress the immune response and play a role in tolerance [23].

1.2.1.2 Pro-inflammatory response during sepsis

While a pro-inflammatory response is generally initiated to eliminate pathogens through a variety of reactions, an exaggerated pro-inflammatory reaction likely drives early deaths in sepsis [17]. As mentioned earlier, recognition of microbial products by PRRs on innate immune cells during sepsis leads to activation of transcription factors

such as NF-κB with subsequent transcription of genes, including those encoding cytokines and other inflammatory mediators such as Nitric Oxide (NO). This leads to the release of these mediators by leukocytes, parenchymal cells, endothelial cells, and platelets. The main pro-inflammatory cytokines suggested to be involved in sepsis pathogenesis are Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, IL-6, IL-12, and IL-18 [31]. If control of the inflammatory response is disturbed, the ensuing hyperinflammatory response can result in self-harm to the organism. Blocking or early elimination of these cytokines confers protection in animal models of sepsis, but this has not been translated to human studies where interventions are initiated once the patient has already developed established organ dysfunction [31].

Complement activation is crucial in protective immunity, but its uncontrolled activation can also cause damage to tissues and, ultimately, organ failure. During sepsis, excessive C5a, in particular, is associated with complications associated with MOF [32]. Likewise, the release of Neutrophil Extracellular Traps (NETs), which are composed of DNA, histones, and neutrophil-derived proteinases, can protect the host by trapping and aiding elimination of pathogens. However, they may also contribute to collateral damage and a dysregulated immune response in sepsis [33].

Considering the focus on monocytes in this thesis, several small observational studies have indicated a significant expansion of pro-inflammatory non-classical monocytes in patients with severe sepsis [27, 34-36]. However, the exact proportion and phenotype of monocyte subsets in sepsis appears to vary depending on the nature of infection and conditions that they encounter in blood and tissue. This environment depends on the presence of cytokines, chemokines, tissue metabolites, and other inflammatory mediators [37, 38].

1.2.1.3 Anti-inflammatory response during sepsis

As a pro-inflammatory response in sepsis is mounted, the body simultaneously initiates a counterbalancing anti-inflammatory response, with the release of anti-inflammatory cytokines such as IL-10 [39, 40]. While this anti-inflammatory response is necessary to suppress excessive inflammation, the effector function of circulating immune cells is downregulated, leading to immune suppression [17, 18]. This places patients at increased risk of secondary infection and death, especially if the hypo-immune state is prolonged. This degree of abnormality is often associated with worse outcomes [41].

The immunosuppressive phase of sepsis involves both innate and adaptive immune systems and can by characterised by various changes in their respective immune cells. Main mechanisms include lymphocyte apoptosis and exhaustion, and reprogramming of APCs. Sepsis is associated with a marked depletion of NK cells, $CD4^+$ and $CD8^+$ T-cells, B-cells, and DCs secondary to apoptosis [18, 42]. Inhibition of apoptosis of these cells improved outcomes of sepsis in experimental models, which suggests a causal role of the loss of lymphocytes in sepsis lethality [43, 44]. However, this has yet to be tested in patients.

In addition to depletion of lymphocytes, altered T-cell function can also contribute to decreased lymphocyte function during the immunosuppressive phase of sepsis. This includes suppressed $CD4^+$ Th1, Th2 and Th17 cell function [18]. T-cells isolated from the spleen during post-mortem had lower IFN-γ and TNF-α production and increased expression of checkpoint regulators such as Programmed Cell Death-1 (PD-1) and Cytotoxic T-Lymphocyte-Associated Protein-4 (CTLA-4), which may compromise T-cell function at a local tissue level. Similar effects were seen for B-cells [42, 45]. Expansion of Treg and Myeloid-Derived Suppressor Cells (MDSC) may also contribute to decreased effector T-cell, monocyte, and neutrophil function [46, 47]. This is associated with an increased risk of secondary infection in patients [48, 49].

The most studied biomarker of sepsis-induced immunosuppression is decreased Human Leukocyte Antigen (HLA)-DR expression on monocytes. This is associated with a higher risk of secondary infection and increased mortality [50, 51]. A reduction in HLA-DR expression, increased apoptosis, and increased production of IL-10 has also been observed in DCs from septic patients [52]. Cells of the innate immune system show a decreased capacity to release pro-inflammatory cytokines upon stimulation. Key findings in neutrophils include delayed apoptosis and the appearance of immature band-like neutrophils [53]. These cells have deficits in anti-microbial effector function, including oxidative burst capacity and chemotactic activity [54-56].

1.2.2 Hormonal alterations

Like the inflammatory response, the endocrine response during sepsis and other critical illnesses follows a distinct biphasic pattern. Although acute changes are probably adaptive, they may become maladaptive during the prolonged phase of sepsis. The acute phase is characterised by abrupt and massive release of stress and metabolic hormones, including cortisol, catecholamines, vasopressin, glucagon, and growth hormone. There is a concurrent shutdown of less vital systems such as gonadal function. Anabolism is also inhibited [57].

Another manifestation during sepsis is the induction of insulin resistance, during which normal concentrations of insulin produce a subnormal response, potentially due to downregulation of insulin receptors [58, 59]. In the later phase, after an undefined period of critical illness of hours to days, the hormonal profile alters substantially with concentrations of vasopressin that are inappropriately low, the onset of the ‘sick euthyroid syndrome’, and reduced adrenal responsiveness of Adrenocorticotropic Hormone (ACTH), often despite high cortisol levels [57, 60]. The magnitude of the above hormonal alterations has major prognostic implications [61, 62].

1.2.2.1 Catecholamines

Catecholamines function both as neurotransmitters and as hormones. They are produced from L-tyrosine hydroxylation to L-3,4,Dihydroxyphenylalanine (L-DOPA), and subsequent conversion into dopamine, noradrenaline, and adrenaline. Activation of the sympathetic nervous system leads to release of adrenaline and small amounts of noradrenaline into the circulation from chromaffin cells within the adrenal medulla. Most noradrenaline is released from neurons within the locus coeruleus and lateral tegmental field [63, 64]. Removal is subsequently induced by reuptake into nerve endings, although some spill-over into the bloodstream may occur [65].

Shock states, and critical illness in general, result in very early elevations in both plasma adrenaline and noradrenaline, the magnitude of which is greater in those who go on to die [66]. During prolonged phases of stress and critical illness, catecholamine levels normally normalise, however this is not universal. In paediatric burn patients urinary catecholamines were elevated for up to 2 years [67].

Catecholamines act by binding with variable affinity to α- and β-Adrenergic Receptors (ARs). These receptors and their subclasses are differentially expressed on various tissues and cells. While β-ARs are mainly expressed in the heart, α-ARs are mainly expressed on vascular smooth muscle. However, ARs have also been found on other cells, including those of the pancreas, liver, kidney, and on immune cells [58, 68]. These receptors and their subclasses exert differential effects on cardiovascular and respiratory systems, and on metabolism. While noradrenaline acts mostly on α-ARs, it does also stimulate β-ARs to a certain degree. The affinity of adrenaline is dose dependent, with high affinity for the β_2-AR in low doses and increasing α-AR affinity in higher doses [69].

Activation of the α_1-AR mainly induces vasoconstriction and smooth muscle contraction, while β_2-AR activation induces vasodilatation and smooth muscle relaxation [70]. β_2-AR activation also increases Heart Rate (HR), has inotropic effects,

elevates glucose levels by inducing glycogenolysis, and alters pancreatic secretion of insulin and glucagon [71]. The α_2-AR is involved in decreasing lipolysis, while the β_1-AR has chronotropic and inotropic effects and stimulates Hormone-Sensitive Lipase (HSL), thereby initiating lipolysis [72]. Lastly, the β_3-AR regulates thermogenesis in skeletal muscle and enhances lipolysis [58]. The combination of these effects is usually an increased availability of O_2 and energy substrates to tissues.

1.2.2.2 Cortisol

The Hypothalamus-Pituitary-Adrenal (HPA) axis is responsible for production and secretion of cortisol and Dehydroepiandrosterone (DEHA) by the adrenal glands. Under normal conditions, the hypothalamus secretes Corticotropin-Releasing Hormone (CRH) in a pulsatile manner. This stimulates the anterior pituitary to secrete ACTH, which regulates adrenal production of cortisol and DEHA. Other ACTH effects include regulation of aldosterone production by the renin-angiotensin system. Cortisol induces negative feedback on CRH and ACTH secretion [73].

Glucocorticoid receptors (GRs) are differentially expressed in most cells and tissues, but main target tissues are liver, the vasculature, immune cells, and the hippocampus [58, 74]. These receptors belong to the nuclear receptor superfamily of transcription factors [75]. General effects of cortisol include minimising excessive inflammation, maintaining vascular tone and endothelial and vascular permeability, and regulating metabolism. Their metabolic effects include increasing gluconeogenesis and glycogenolysis, increasing peripheral insulin resistance, and increasing free fatty acids and amino acids [58, 73, 76].

During acute stress, the HPA axis is activated by neuronal circuits and release of inflammatory cytokines including TNF-α, IL-1 and IL-6 [77]. Vasopressin, endothelin, Atrial Natriuretic Factor (ANF), and Macrophage-Inhibiting Factor (MIF) are other stimulators. These additive effects result in high levels of cortisol during critical illness,

but normal diurnal variation is lost [78-80]. Despite variations in cortisol levels related to age, the degree of this increase reflects the severity of illness [61].

Despite high cortisol levels, plasma levels of ACTH are often low. Impaired glucocorticoid clearance is therefore thought to be a main contributor to high cortisol levels in critical illness [81]. In addition, excessive cytokine production may reduce the number and binding affinity of GRs [82]. However, both increases and suppression of GR number and sensitivity have been reported [82, 83]. A reduction in GR number and binding affinity during sepsis could lead to glucocorticoid resistance.

During the chronic protracted phase of critical illness, circulating cortisol levels remain high [84]. Diminished cortisol breakdown becomes more prominent in increasing cortisol levels, as the cortisol production rate is only moderately increased beyond the very acute phase [81]. Harmful complications of these high cortisol levels include hyperglycaemia, myopathy, and increased susceptibility to infection [73]. Despite hypercortisolaemia, reduced adrenal responsiveness to ACTH is often observed during prolonged sepsis [85, 86].

1.2.2.3 Thyroid hormones

Thyroid hormone production is regulated by the Hypothalamus-Pituitary-Thyroid (HPT) axis. Secretion of hypothalamic Thyroid-Releasing Hormone (TRH) stimulates pituitary production and release of Thyroid-Stimulating Hormone (TSH). TSH is released in bursts and activates the thyroid gland to produce Thyroxine (T_4). This hormone is peripherally converted by deiodinases into its active form Free Triiodothyronine (fT_3) and metabolically inactive Reverse T_3 (rT_3). Both hormones exert negative feedback on TRH and TSH production by the hypothalamus and anterior pituitary, respectively. The majority of thyroid hormone in plasma is protein-bound to Thyroxine-Binding Globulin (TBG), Transthyretin (TTR), or albumin, and therefore inactive.

The Thyroid hormone Receptor (TR) is a nuclear receptor expressed on a wide variety of cell types and tissues, including immune cells [87]. Thyroid hormones are essential inducers of energy metabolism, cell differentiation, and growth [88]. Their metabolic effects include increased carbohydrate and fat metabolism, and reduced plasma levels of cholesterol, phospholipids and triglycerides [58].

During the acute phase of critical illness, circulating levels of T_3 decrease, whereas levels of rT_3 increase. The degree of change reflects the severity of illness [89]. After a prolonged phase of critical illness, levels of T_3 further decline and T_4 levels are also reduced. This phenomenon is known variously as the 'sick euthyroid syndrome', 'low T_3 syndrome', or 'Non-Thyroidal Illness' (NTI) [90, 91].

Altered peripheral conversion of T_4 is likely responsible for these acute changes. Levels of T_4 increase briefly and return to normal though, occasionally, T_4 can decrease in very severe acute disease. These changes aim to protect the organism by reducing energy consumption and catabolism, and may be beneficial during the acute phase. While TSH levels quickly decrease to the normal range, the nocturnal TSH surge is absent [90, 91]. Other features include reduced concentrations of thyroid hormone binding proteins, inhibition of hormone binding, and changes in hormone transport [90, 91]

During a prolonged period of time, pulsatile TSH secretion becomes suppressed, which correlates with suppressed hypothalamic TRH gene expression [90]. The combination of low peripheral thyroid hormone, together with low TSH and TRH, suggests major changes in the central regulation of the HPT axis. Triggers for these changes remain unclear, but it could possibly be induced by cytokines, sustained hypercortisolism, neuropeptide Y, or alterations in hypothalamic deiodinase activity and transporter expression. Treatment with dopamine and corticosteroids could also play a role [90-92].

1.2.2.4 Glucagon

Glucagon is a catabolic peptide hormone, secreted by α-cells of the islets of Langerhans within the pancreas in response to a decreased availability of energy substrates. It has a catabolic function that counteracts the effects of insulin by increasing synthesis and release of glucose, fatty acids, and ketone bodies into the circulation through glycogenolysis, gluconeogenesis, ketogenesis, and lipolysis. It also regulates urea synthesis. Glucagon is often studied in conjunction with insulin. An increased glucagon to insulin ratio is responsible for a fuel switch from glucose to free fatty acids in the liver [93].

The primary pathway of action involves binding of glucagon to a G-protein coupled trans-membrane receptor with formation of Cyclic Adenosine Monophosphate (cAMP) through activation of adenylate cyclase. cAMP is a second messenger activating the Protein Kinase A (PKA) pathway that phosphorylates and (de)activates various metabolic enzymes. An important second messenger of glucagon is Inositol Triphosphate (IP_3), which leads to Ca^{2+} release from the Endoplasmic Reticulum (ER), increasing intracellular Ca^{2+} levels [94, 95].

Glucagon rises in acute illness to oppose the actions of insulin. It also plays a role in elevating sugar levels and other metabolic fuel substrates [71]. Its receptor has been found in many tissues, but is mainly expressed in the liver, where it increases plasma glucose levels via glycogenolysis and gluconeogenesis [58]. In patients undergoing emergency laparotomy for abdominal sepsis, glucagon levels were elevated in those patients who later died from MOF compared to survivors [96]. Glucagon levels may reflect disease severity and clinical outcomes in septic patients [97].

1.2.2.5 Insulin

This anabolic hormone is secreted by β-islet cells within the pancreas and corresponds to changes in energy metabolism [98]. It is released from secretory granules following an increase in intracellular Ca^{2+} or cAMP signalling. Insulin and glucagon counteract each other in response to the availability of energy substrates. Insulin binds to the insulin receptor, a transmembrane receptor within the class of tyrosine kinase receptors [99]. This receptor is expressed in most cells, but mainly in liver, skeletal muscle, and fat [100]. Effects include promoting glucose entry into cells, decreasing gluconeogenesis, proteinolysis and lipolysis, and increasing fatty acid and glycogen synthesis [58]. This ultimately results in increased storage of energy substrates.

Insulin may fall transiently during early sepsis, resulting in an increased availability of metabolic fuel substrates. This is thought to be caused by increased clearance rather than decreased secretion [101]. Non-pulsatile and insufficient insulin secretion follow, as well as the development of insulin resistance which manifests itself as hyperglycaemia and hyperinsulinaemia. This phenomenon can partially be explained by the effects of pro-inflammatory cytokines, and is amplified even further by other endogenous stress hormones and drugs such as catecholamines and corticosteroids [58, 59, 102]. Insulin sensitivity provides a negative predictive diagnostic for sepsis, with higher degrees of insulin resistance being associated with higher mortality and organ dysfunction [103].

1.2.3 Mitochondrial (dys)function

The ultimate cause of death in most patients with sepsis is MOF. Although the precise pathophysiology leading to this phenomenon remains uncertain, it appears to relate to metabolic dysfunction rather than structural damage, as (i) no or minimal structural damage is found in affected organs sufficient to account for the organ dysfunction

[104], (ii) O_2 remains available to the organ but is not utilised [105], and (iii) affected organs normally recover in surviving patients [105, 106]. A metabolic shutdown, driven by a lack of sufficient Adenosine Triphosphate (ATP) generated by mitochondria, could well explain this phenomenon. Indeed, Reduced Oxidative Phosphorylation (OXPHOS) is associated with sepsis-induced MOF [107].

1.2.3.1 Mitochondrial function in health

Mitochondria are organelles that reside within the cytoplasm of virtually all cell types, albeit in variable numbers. They consist of outer and inner membranes, with an intermembrane space in between, and a mitochondrial matrix within the inner membrane (Figure 2). In most cells, relatively small amounts of ATP are produced in the cytosol via glycolysis, which is independent of O_2, but most is produced by mitochondria through OXPHOS. In addition to energy production, mitochondria exhibit a plethora of other functions, including production of Reactive Oxygen (ROS) and Nitrogen (RNS) species, induction of thermogenesis, regulation of Ca^{2+} signalling, apoptosis and cell death, and steroid hormone metabolism.

The inner membrane holds the transporters Coenzyme Q10 and cytochrome C, and five enzyme complexes (Complexes I-V) that comprise the Electron Transport Chain (ETC). The Tricarboxylic Acid (TCA) cycle, also known as citric acid cycle, is located within the mitochondrial matrix. Reduced Nicotinamide Adenine Dinucleotide (NADH) and Flavin Adenine Dinucleotide ($FADH_2$) are derived from acetyl-CoA within the TCA cycle. While amino acids may directly enter the cycle, glucose and fatty acids first need to be converted into acetyl-CoA via glycolysis and β-oxidation. Electrons are then transferred from NADH and $FADH_2$ to the ETC, which donate their electrons to Complexes I and II, respectively.

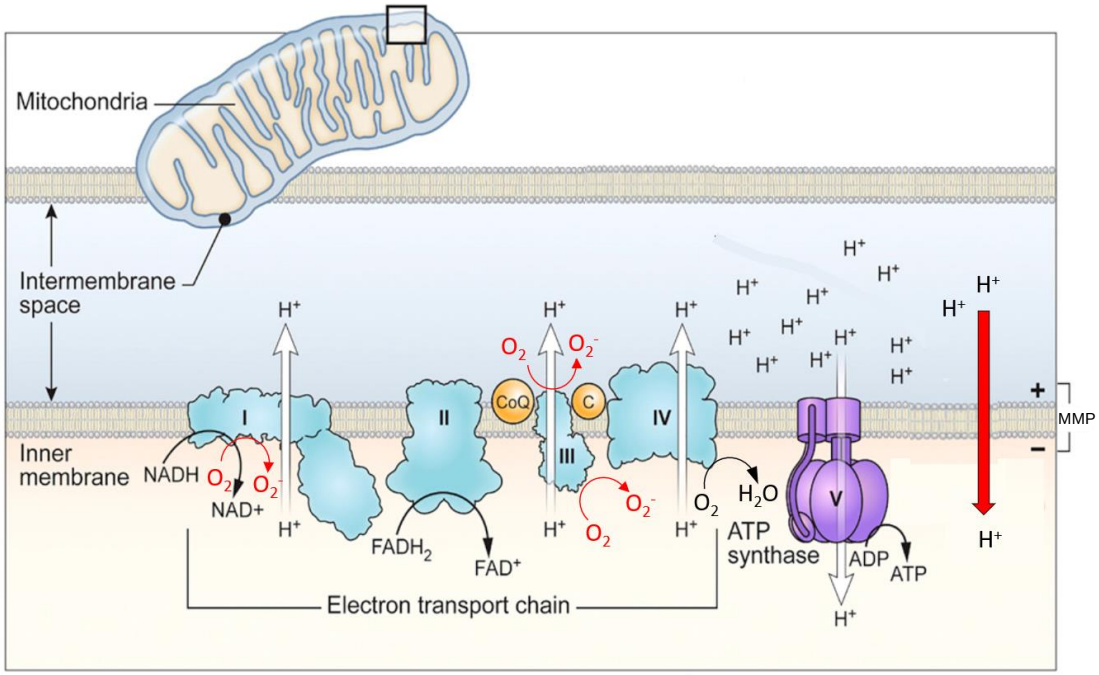

Figure 2. Mitochondrial ETC and complexes. NADH and $FADH_2$ donate electrons to Complex I and II of the ETC. These electrons move down the chain while protons are being pumped into the intermembrane space creating a proton gradient that is used by Complex V (ATP synthase) to form ATP from ADP. O_2 functions as the final electron acceptor at Complex IV and is reduced to water. Protons can also move back into the inner membrane space by proton leak or uncoupling. Premature or incomplete reduction of O_2 will produce O_2^- at Complexes I and III. ADP: Adenosine Diphosphate; ATP: Adenosine Triphosphate; C: Cytochrome C; CoQ: Coenzyme Q10; FAD(H_2): Flavin Adenine Dinucleotide; MMP: Mitochondrial Membrane Potential; NAD(H): Nicotinamide Adenine Dinucleotide; O_2^-: Superoxide. Adapted from [108].

As these electrons move down the chain through the first four enzyme complexes, protons are pumped from the mitochondrial matrix into the intermembrane space. At Complex IV, O_2 functions as the final electron acceptor, and is reduced to water. Mitochondria use most of the body's O_2 consumption for this process. Due to the impermeability of the Inner Mitochondrial Membrane (IMM), protons cannot easily move back into the matrix and therefore create a proton gradient. This gradient is used by Complex V (ATP synthase) to phosphorylate ADP to ATP. Adenosine Nucleotide Transferase (ANT) assists ATP/ADP exchange across the mitochondrial

membrane, transporting ATP out of the mitochondria into the cytosol to fuel metabolic processes [109, 110].

In most cells, the majority of ROS is produced as a by-product of OXPHOS within mitochondria. In immune cells, ROS can also be produced by NADH Phosphate (NADPH) oxidase [111]. Free electrons play a significant role in ROS generation. Premature or incomplete reduction of O_2 will produce superoxide radicals, predominantly at Complex III, but also at Complex I. This is a natural by-product of respiration; in health it accounts for 0.2-2% of molecular O_2 consumption [112].

ROS plays an important role in signalling, immunomodulation, vascular tone maintenance, and O_2 sensing [109]. Whereas regulated Mitochondrial ROS (mROS) functions as a signalling molecule and contributes to bacterial killing in small concentrations, excessive production can overwhelm antioxidant defences and lead to oxidative stress by damaging proteins, membrane lipids, and nucleic acids, resulting in DNA damage [111, 113, 114]. Mitochondrial antioxidants such as Manganese Superoxide Dismutase (MnSOD) and Glutathione (GSH) protect mitochondria from this mROS-induced damage.

The Mitochondrial Membrane Potential (MMP) may be decreased by uncoupling and proton leak, whereby protons move back across the IMM without going through ATP synthase. The energy is transferred to heat (thermogenesis) [115]. Inducible proton leak or uncoupling is mediated via Uncoupling Proteins (UCPs). Five different UCPs have been found in humans with differential expression in various tissues [115-117]. Proton leak and uncoupling may represent a protective mechanism to decrease mROS production and protect mitochondria from oxidative damage [118]. This mechanism can be induced by ROS, lending further support to a protective role for thermogenesis [119]. The leak of protons is closely linked to MMP; a higher MMP increases proton leak, while a raised proton leak rate increases O_2 consumption and decreases MMP [120].

Another mitochondrial mechanism that modulates the complex interaction between mROS and mitochondrial function is the Mitochondrial Permeability Transition Pore (mPTP). This transmembrane protein is normally closed, but opens with Ca^{2+} overload, excessive ROS or RNS, adenine nucleotide depletion, and loss of the MMP [121]. Its opening is often referred to as a pathological event, causing mitochondrial depolarisation, disruption of OXPHOS, Ca^{2+} release, and matrix swelling. mPTP opening may identify dysfunctional mitochondria to undergo selective autophagy, leading to an increased turnover and production (biogenesis) of healthy mitochondria [122]. It may also act as a checkpoint, integrating energy metabolism with cell death pathways [123]. Processes induced by mPTP opening can result in ATP depletion, outer mitochondrial membrane damage, and initiation of intrinsic mitochondrial apoptosis [124].

All these physiological roles of mitochondria make it a highly diverse and active organelle. Quality control is therefore crucial to ensure performance. The production and replacement of mitochondrial proteins, mitochondrial biogenesis, encoded by nuclear or mitochondrial DNA, improves mitochondrial capacity for energy production. This is orchestrated by PPAR-γ Coactivator 1α (PGC-1α) which activates transcription factors such as Nuclear Respiratory Factors (NRF)-1 and 2, and Transcription Factor A for the Mitochondrion (Tfam) [125].

Mitochondria also undergo morphological changes during fusion and fission. These events are important for cell division and proliferation, and the removal of damaged mitochondria by mitophagy. Proteins driving fusion and fission events have been associated with altered MMP and reduced O_2 consumption, highlighting that these separate mitochondrial functions are highly interactive [126].

1.2.3.2 Mitochondrial dysfunction in sepsis

Mitochondria are affected during sepsis by impaired perfusion, leading to tissue hypoxia, excess amounts of NO and ROS, hormonal alterations, and regulation of genes transcribing mitochondrial proteins [109]. These effects cause inhibition, damage, or decreased turnover of mitochondrial proteins, and affect the generation of ATP. Mechanisms to cope with this decreased energy substrate supply include switching to non-mitochondrial ATP production through glycolysis, or decreasing metabolic activity to reduce energy requirements akin to hibernation [127, 128]. Excellent reviews on mitochondrial dysfunction during sepsis in a variety of cells and tissues, including immune cells, brain, heart, kidney, and liver have already been published [128, 129]. An overview of the main changes is given below.

A landmark 2002 publication from our group showed mitochondrial dysfunction in skeletal muscle from patients with septic shock. A correlation was seen between the degree of mitochondrial dysfunction (reduced activity of Complex I and ATP depletion), increased NO production, decreased concentration of the mitochondrial antioxidant GSH, and illness severity and outcome [107]. Similar results were obtained from a long-term animal model of sepsis by our group [130]. Numerous others have subsequently investigated the role of mitochondria in sepsis. Though some evidence is conflicting, this can likely be explained by inconsistencies in models and techniques used, but also by timing, tissue specificity, and disease severity [129].

The adverse effects of sepsis on mitochondria are manifested in several ways, including structural damage, respiratory chain and OXPHOS abnormalities, uncoupling, oxidative and nitrosative stress, induction of the intrinsic apoptosis pathway, and altered mitochondrial dynamics, mitophagy, and biogenesis.

Reduced respiratory complex expression and activity, especially of Complexes I, III and IV has been reported in muscle from septic patients [131-133]. Mitochondrial activity of circulating white blood cells and platelets has also been studied extensively

as they are readily accessible. For example, mitochondrial depolarisation during apoptosis has been detected in septic monocytes and correlates with mortality [134, 135].

However, mitochondrial changes in different cell types are not universal. For example, effects in skeletal muscle are not like those observed in platelets. Induced respiratory capacity has been observed in platelets from septic patients with simultaneous augmented leak respiration. This suggests defective coupling of the respiratory chain and was negatively associated with clinical outcomes [136, 137]. Increased proton leak and expression of UCPs have been reported during sepsis in several tissues [137-142]. Complex IV activity from platelets is decreased in sepsis but higher in survivors; this is associated with clinical outcomes [143, 144].

Increased mitochondrial respiratory capacity has been observed in Peripheral Blood Mononuclear Cells (PBMCs) during sepsis, again likely due to increased leak respiration and uncoupling, disconnecting respiration from ATP synthesis [145-148]. This phenomenon is associated with clinical outcomes as coupled respiration was lowest in non-survivors [149]. By contrast, increased activity of Complex I and IV in monocytes has also been reported in sepsis [150]. In a recent publication, it was concluded that mitochondrial measurements from PBMCs varied with changes in immune cell composition. However, differences between sepsis and controls were still partly attributable to the effects of sepsis [151].

Uncoupling of OXPHOS generally results in a more oxidative redox state at the site of electron leak and, consequently, a decrease in mROS production. Despite the initiation of this likely protective mechanism during sepsis, increased ROS generation and oxidative stress are reported in a wide variety of cells and tissues, contributing to organ failure [113, 152, 153]. Sepsis-induced oxidative stress is linked to the induction of mitochondrial respiratory dysfunction [154-156]. Increased production of NO also inhibits the ETC, especially Complex IV [107, 157, 158]. NO can react with superoxide

to form the highly reactive peroxynitrite, which can inhibit other mitochondrial complexes, in particular Complex I [159-161].

Both excessive ROS production and a decreased antioxidant defence contribute to oxidative stress during sepsis. A reduced antioxidant capacity, including falls in coenzyme Q10 and MnSOD, are associated with mitochondria-related organ failure and worse outcomes in sepsis [107, 162-165]. Clinical trials with antioxidants are however conflicting [166, 167]. Excess levels of MnSOD may increase hydrogen peroxide and amplify mitochondrial dysfunction [168]. This highlights the challenge to selectively reduce pathological levels of mROS, while maintaining adequate levels for cell signalling and immune function.

1.2.4 Metabolic changes

As with most responses during sepsis, metabolism is also recognised by a biphasic pattern. An initial acute catabolic phase is driven by the release of stress hormones and pro-inflammatory cytokines, leading to the rapid mobilisation of energy stores. This includes the induction of gluconeogenesis with breakdown of protein from muscle, breakdown of glycogen stores, and induction of lipolysis [169]. The accompanying rise in glucose levels can lead to hyperglycaemia, which has been associated with increased mortality [170-172]. Tight glycaemic control using insulin has been argued as a beneficial treatment, however, variable outcomes in studies make this strategy an ongoing topic of debate [89].

Despite the increased availability of energy substrates during the acute phase, lactate is often produced from pyruvate by lactate dehydrogenase for energy generation under conditions of insufficient O_2 supply. Blood lactate has been used as a marker for systemic tissue hypoperfusion and is a criterion for septic shock [2]. During the acute phase, patients often have decreased food intake and thus accumulate a rapidly evolving energy deficit [173, 174]. However, as the body subsequently enters a state

of hibernation with reduced metabolism, the caloric need does not increase consistently [175].

This cellular metabolic downregulation may well represent an adaptive response to reprioritise energy consumption and limit additional injury, maintain energy balance, prevent DNA damage, and preserve cellular composition [176]. Cellular metabolic reprogramming during sepsis may be orchestrated by several coordinated programs, including shifts in metabolic ATP generation, inhibition of mitochondrial respiration, activation of quality-control mitochondrial processes, and the induction of cell cycle arrest. Contrary to the intermediate hibernation phase, the late recovery phase is characterised by another hypermetabolic response, with increased O_2 and substrate utilisation [175, 177]. Failure to restore OXPHOS can perpetuate a pro-inflammatory state that limits organ function and survival [178].

Immune cells undergo changes in cellular metabolism upon inflammatory stimulation, correlating with effector function, polarisation, and survival [179]. A shift from OXPHOS to glycolysis (Warburg effect) is important for these cells to generate an inflammatory response upon stimulation. This requires preferential oxidation of glucose through glycolysis, despite the availability of O_2, albeit less efficient in producing ATP than OXPHOS. While endotoxin induces a classical Warburg effect, various bacterial stimuli induced a rise in both glycolysis and OXPHOS in monocytes [180]. A disturbed balance in cellular metabolic processes has been implicated in the altered phenotype of monocytes in sepsis.

The dependency on these metabolic pathways varies between immune cells. Neutrophils predominantly rely on glycolysis, whereas monocytes, macrophages, and lymphocytes reprogram so that housekeeping functions are sustained through OXPHOS, but energy substrates required for activation are derived from glycolysis [181, 182]. At sites of inflammation with low glucose availability, monocytes can also upregulate fatty acid oxidation and thus OXPHOS [183]. Induction of glycolysis has

two advantages for immune cells: i) production of essential structural components, such as fatty acids, amino acids, and nucleotides, and ii) shunting of glycolytic intermediaries through the pentose phosphate pathway, thereby increasing NADPH, which is key to reducing oxidative damage from mROS [184].

Therefore, it would be interesting to see how metabolic downregulation with inhibition of mitochondrial energy production and function contributes to dysregulation of energy-demanding immune cell effector functions, such as cytokine production and HLA-DR expression on monocytes during sepsis, ultimately leading to higher mortality rates.

1.3 Endocrine-induced effects in health and pathology

1.3.1 Endocrine-induced effects on mitochondria

1.3.1.1 Catecholamines

Catecholamines have a marked effect on energy metabolism, accelerating aerobic glycolysis and increasing O_2 demands [185]. They provide precursor molecules for energy production by releasing lactate from skeletal muscle, breaking down amino acids, glycogen, and triglycerides, and enhancing glucose release from glycogenolysis and gluconeogenesis. Together with the inhibition of insulin-induced glycogenesis, this results in the generation of glucose, fatty acids, and ketone bodies, and induces hyperglycaemia and hyperlactatemia [186-188]. These catecholamine-induced effects are mainly β_2-AR mediated [189].

Effects on mitochondrial activity are however conflicting. This can most likely be explained by differences in patients and animals, timing, tissue specificity, and dosing. Catecholamines induced an acute increase in OXPHOS coupling, O_2 consumption, and ATP production in rat liver mitochondria, although their long-term effects are unknown [190]. Similarly, noradrenaline stimulated hepatic succinate dehydrogenase

through β-AR activation [191]. Other catecholamines, including dobutamine and dopamine, increased respiration efficiency in mitochondria from pig skeletal muscle [192]. More specifically in immune cells, in catecholamine-trained primary human monocytes, Lipopolysaccharide (LPS) with co-incubation with noradrenaline and adrenaline both increased O_2 consumption and Extracellular Acidification Rates (ECAR) – a measure of glycolysis – after 6 days [193].

By contrast, catecholamine-induced hypermetabolism may also promote mitochondrial dysfunction, by enhancing oxidative stress via accelerating glycolytic pathways or through catecholamine auto-oxidation [194]. In line with this, noradrenaline and adrenaline acutely inhibited O_2 consumption and ECAR in human PBMCs and monocytes [193, 195, 196]. Similarly, mitochondrial function was impaired in isolated liver mitochondria from endotoxic pigs treated with dopamine, dobutamine or noradrenaline [197]. Although not mitochondria-specific, noradrenaline also impaired ROS production by monocytes and neutrophils [196]. In rats, isoprenaline promoted cardiac mitochondrial dysfunction by opening of the mPTP and increased membrane swelling [198].

1.3.1.2 Cortisol

Glucocorticoids mobilise energy substrates such as glucose and lipids into the circulation for ready access by organs under stress. This may result in hyperglycaemia which can, in itself, cause damage to mitochondria [199]. Regulation of mitochondrial energy metabolism by glucocorticoids is induced by rapid non-genomic activation of kinase signalling pathways [200, 201], as well as by direct effects on both nuclear and mitochondrial gene expression [202]. Direct effects on the mitochondrial genome are regulated by translocation of GRs into the mitochondria, which subsequently bind to Glucocorticoid Response Elements (GREs) [203, 204]. Glucocorticoid chaperones and the mitochondrial translocation machinery are

important regulators of GR import into mitochondria, affecting their function and survival [205].

GRs can also modulate gene expression independently by interacting with transcription factors [206]. These effects are associated with changes of mitochondrial-encoded OXPHOS genes, thus influencing mitochondrial function in parallel with nuclear and non-genomic actions. Mitochondrial metabolism seems to be affected by glucocorticoids in a biphasic manner, depending on treatment dose and exposure duration. Differential effects might be explained by concentration-dependent effects on both GRs and Mineralocorticoid Receptors (MRs) [207]. Glucocorticoids induce a dose-dependent association of GRs with the mitochondrial genome. High glucocorticoid levels result in lower GR binding compared to moderate levels, resulting in concomitant changes in Mitochondrial RNA (mtRNA) gene expression, as enhanced mitochondrial gene expression normally augments energy production capacity [208-210].

Whereas short-term exposure to glucocorticoids serves as a protective mechanism, long-term exposure could have deleterious effects on mitochondrial function. For example, short-term treatment of primary nerve cells with high or low doses of corticosterone, and long-term treatment with low doses, enhanced mitochondrial oxidation, MMP, Ca^{2+} buffering capacity, and resistance to apoptotic signalling, while long-term treatment with high doses produced inhibition [211]. These rapid short-term effects were associated with non-genomic activation of the Protein 38 (p38)-Mitogen-Activated Protein Kinase (MAPK) pathway, while long-term effects were associated with genomic actions causing a decrease in OXPHOS efficiency [212].

As evidenced by other studies, acute and limited corticosteroid exposure stimulates mitochondrial capacity and energy generation through activation of respiratory chain components, enhancement of mitochondrial and nuclear gene expression, Ca^{2+} accumulation, increases in MMP, prevention of programmed cell death, and

increased mitochondrial biogenesis and mtDNA content [213-223]. These effects ultimately result in increased cellular energy capacity.

By contrast, long-term exposure causes respiratory chain dysfunction, decreased ATP production, increased ROS generation, mitochondrial structural abnormalities, abnormal mitochondrial biogenesis, decreased MMP, and increased sensitivity to cell death [199, 224-227]. Glucocorticoids reduced the Respiratory Control Ratio (RCR) through inhibition of Complex IV activity in isolated rat kidney mitochondria, as well as through inhibition of Complex I and V activity in rat brain mitochondria [228, 229]. Ca^{2+} influx in myocytes is also inhibited [230].

In addition to the abovementioned effects, both GRs and MRs participate in the control of energy substrate production through inhibition of UCP1 and UCP3 in Brown Adipose Tissue (BAT) [231]. Glucocorticoids can also cause mitochondrial fragmentation in hepatocytes by inducing proteins that promote mitochondrial fission [232]. They also regulate apoptosis via the intrinsic pathway. However, the direction of effects again appears to be both tissue- and dose-dependent [233, 234]. For example, long-term exposure to high concentrations of corticosterone reduced mitochondrial localisation of the GR and decreased the formation of GR with anti-apoptotic proteins in neuronal cells, whereas short-term exposure with physiological concentrations enhanced mitochondrial GR/anti-apoptotic protein induction [211].

1.3.1.3 Thyroid hormones

Thyroid hormones are major regulators of mitochondrial biogenesis and mitochondrial activity. In contrast to the traditional thyroid hormones, rT_3 displays hypometabolic properties and could antagonise hypermetabolic effects [235]. These effects are thought to be mediated through genomic actions, altering the expression of nuclear and mitochondrial genes, rather than direct or non-genomic effects on membranes.

Importantly, when assessing the effects of thyroid hormones on mitochondrial function, one must keep in mind that supraphysiological doses are often used [236].

Genomic actions of thyroid hormones are the result of a complex interaction between nuclear and mitochondrial genomes, where Thyroid Response Elements (TRE) interact with TRs [237-241]. This affects target genes and intermediate transcription factors and co-activators [219, 242, 243]. For example, induction of PGC-1α has profound effects on mitochondrial biogenesis, metabolic rate, and thermogenesis [244]. Additionally, rapid non-genomic actions of thyroid hormone signaling involve cytoplasmic kinases such as MAPK and Phosphoinositide 3-Kinase (P13K) which, in some cases, have downstream effects on gene transcription [245-249]. Hyperthyroid states increase mitochondrial O_2 consumption due to increased import and oxidation of fuel substrates, facilitated by increased expression and activity of oxidative enzymes [250-252].

This increased O_2 consumption may induce increased ATP synthesis, but it also appears that much of the energy from oxidation may be lost as heat rather than being used for ATP production, due to increased proton leak and uncoupling [253, 254]. Hypermetabolic and uncoupling effects of thyroid hormones have been long recognised [255, 256], but precise underlying mechanisms are still debated. These include activity of membrane carrier proteins such as ANT [257], UCPs [258-260], changes in phospholipid composition of the IMM [261-263], and induction of mPTP opening [264].

Uncoupling can, as explained previously, limit mROS production. Underlying mechanisms of increased oxidative damage by thyroid hormones include alterations in ROS production and changes in antioxidant defence, including falls in MnSOD and GSH [265]. Hypothyroidism results in decreased mitochondrial hydrogen peroxide, and other markers of oxidative stress, including decreased lipid peroxides and DNA damage [266, 267]. However, the effects of hyperthyroidism are controversial.

Increased ROS damage, particularly in highly oxidative tissues such as muscle and heart, may be due to augmented oxidative metabolism and decreased antioxidant activity [267]. By contrast, no changes in ROS production were found in cardiac tissue by others, potentially due to increased uncoupled respiration [266].

Other effects of thyroid hormones on mitochondrial function include regulation of Ca^{2+}, which acts as a second messenger through modulation of Ca^{2+}-ATPase [268, 269]. Hyperthyroid mitochondria have a greater Ca^{2+} content, which could increase coupled respiration by activation of mitochondrial dehydrogenase [270]. In addition, thyroid hormones regulate mitochondrial quality control in liver and skeletal muscle by induction of autophagy [271, 272].

1.3.1.4 Glucagon

Effects of glucagon on mitochondrial function have been studied predominantly in the liver, as this is a highly metabolic organ regulating energy substrate levels. Glucagon stimulates respiration, mitochondrial enzyme activity, and ATP synthesis, and increases MMP in liver, BAT, and brain mitochondria [273-285]. However, the RCR was not affected by glucagon in brain mitochondria [281]. An increased glucagon to insulin ratio is responsible for a decrease in glycolysis and increase in OXPHOS [286].

Potential mechanisms include induced uncoupling, increased Ca^{2+}, an increase in mitochondrial adenine nucleotide content, and regulation of transcription factors and gene expression. While Yamazaki et al. (1975) reported that increased respiration after glucagon treatment was not caused by uncoupling, this has been shown in BAT and White Adipose Tissue (WAT), muscle, and liver mitochondria [287-290]. However, it is questionable whether effects on BAT and WAT are due to a direct effect of glucagon or an indirect effect of released free fatty acids [290]. Glucagon can also activate Ca^{2+}-sensitive metabolic oxidative enzymes, regulating respiration and

increasing mitochondrial efficiency [291-293]. However, by contrast, others found that glucagon-induced respiration was Ca^{2+}-independent [294].

Other potential explanations for glucagon-induced metabolic effects include regulation of transcription factors with activation of the Extracellular Signal-Regulated Kinase (ERK)1/2 pathway, leading to cAMP-Response Element Binding Protein (CREB) activation and enhanced expression of PGC-1α with upregulated transcription of metabolic genes. Long-term glucagon exposure may however suppress key enzymes regulating mitochondrial function, such as Complex III and IV, and biogenesis [295]. Glucagon may also induce the production of Sirtuins (SIRT), which are associated with mitochondrial function and ATP production [296, 297]. Lastly, glucagon induces autophagy to enhance turnover of mitochondria [298, 299].

1.3.1.5 Insulin

Insulin regulates fuel metabolism via the Insulin Receptor Substrate (IRS)-P13K-Akt signalling pathway with downstream targets Forkhead Box O1 (FOXO1) and Mammalian Target of Rapamycin (mTOR). It is long known that insulin augments O_2 consumption and ATP formation by muscle and liver mitochondria [300-303]. Insulin also modulates mitochondrial oxidative enzyme expression and activity, oxidative damage, and mitochondrial biogenesis. These effects have been evidenced in various tissues, including muscle, liver, heart, neuronal, and pancreatic β-cells, although insulin effects are most often studied in models of insulin resistance or diabetes. Mitochondrial dysfunction in insulin resistance is well recognised [304].

Enhanced muscle mitochondrial ATP production capacity by insulin is associated with increased mRNA expression and activity of mitochondrial and nuclear oxidative enzymes, including Complexes I and IV [305-310]. Insulin deficiency resulted in decreased mitochondrial ATP production and downregulated OXPHOS genes,

especially of Complexes III and IV [311, 312]. Regulation of mitochondrial protein synthesis by insulin may be tissue specific [313].

Insulin may also be involved in regulating mitochondrial biogenesis and uncoupling [314], but data are conflicting [315, 316]. Mitochondrial uncoupling and reduced oxidative capacity were however seen when myocardial insulin signalling was impaired [317]. By contrast, improved mitochondrial function and attenuated proton leak by insulin was found in skeletal muscle cells [318].

This potential regulation of proton leak and uncoupling could diminish oxidative damage. Indeed, mitochondrial oxidative damage is a common feature of insulin resistance [319]. Boudina and colleagues (2009) found that impaired myocardial insulin signalling promoted oxidative stress [317]. Similarly, loss of insulin/Insulin Growth Factor (IGF)-1 signalling in muscle decreased mitochondrial respiration but increased ROS production [320]. Insulin increased antioxidant defences in diabetic mouse cardiomyocytes [321] and in brain [322]. Insulin may also mitigate oxidative stress by minimising glucose-mediated ROS production [79, 323].

While acute low and high doses of insulin increased the expression and activity of OXPHOS proteins in rats, chronic exposure had variable effects. At low dose, insulin decreased Complex I and ATP synthase expression, but increased Complex II and IV expression, and Complex IV activity. However, high dose insulin decreased Complex II and III expression and ATP synthase activity, but increased Complex IV. Expression of UCP1 was also decreased after acute high doses, but increased with chronic exposure [324]. The role of insulin in BAT thermogenesis was also reported by others [325-327]. Golic and colleagues (2020) also found that chronic dosing decreased cytochrome C expression, a mitochondrial protein released into the cytosol during apoptosis [324]. This suggests a protective effect of insulin against the induction of apoptosis. Insulin signalling has additionally shown to regulate myocardial autophagy flux [328].

1.3.2 Endocrine-induced effects on immune cells

1.3.2.1 Catecholamines

For this thesis, I have mainly focussed on the immunomodulating properties of two catecholamines, being adrenaline and noradrenaline. *In vitro* animal and human models reveal immunomodulating effects, albeit with an inconsistent direction [193]. Whereas some pro-inflammatory effects have been reported, most studies indicate immunosuppressive effects. These differences could potentially be explained by differential α- or β-AR activation or training [193, 196]. The Pickkers group have recently published several original and review articles on this topic, a summary of which is included in the overview below.

Activation of the α-AR by noradrenaline results in NF-κB activation with subsequent production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [329, 330]. Conflicting anti-inflammatory effects have also been reported [331]. Of note, conflicting immunomodulatory properties after stimulation have also been found between acute exposure versus catecholamine-trained cells. While both adrenaline and noradrenaline acutely decreased pro-inflammatory cytokine production in an *in vitro* LPS model using whole blood and monocytes, re-stimulation of catecholamine-trained monocytes with LPS after 6 days increased TNF-α and IL-6 production via the β-AR-cAMP pathway [193].

The immunomodulatory effects of noradrenaline appear mainly mediated by β_2-AR activation with subsequent inhibition of NF-κB [330]. Induction of this receptor by noradrenaline results in anti-inflammatory IL-10 production via cAMP and PKA pathways [332]. Other anti-inflammatory effects seen *in vitro* include decreased TNF-α and IL-6 production, diminished NK cell cytotoxicity, downregulation of IL-2 production by Th2 cells, and promotion of bacterial growth [333-336]. *In vivo* animal experiments show comparable results, with increased levels of IL-10 and IL-1β, the induction of an immunosuppressive phenotype in neutrophils, and increased bacterial

growth; susceptibility to infections is thus increased [337-339]. These effects were reversed by both β-blockers and loss of noradrenergic nerve endings [340, 341].

In a recent human endotoxin model, noradrenaline decreased pro-inflammatory IFN-γ Inducible Protein (IP-10), and increased IL-10 levels. In septic patients, higher infusion rates were correlated with a more anti-inflammatory cytokine balance, whereas use of β-blockers was associated with a more pro-inflammatory cytokine balance. *In vitro* and animal models using noradrenaline and LPS similarly decreased monocyte production of pro-inflammatory cytokines and ROS, and increased IL-10 production [196]. The noradrenaline concentrations used in this *in vitro* study however far exceed levels found in the circulation of septic patients receiving a noradrenaline infusion (max. 10 ng/mL) [342]. In addition, noradrenaline infusion in animal experiments was begun before injection of LPS, making translation to the clinical situation challenging.

Similar to noradrenaline-induced effects, adrenaline also decreased production of pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6, IL-8), and increased anti-inflammatory IL-10 in *in vitro* and *ex vivo* models [343-346]. Comparable results were obtained from human endotoxaemia models, where adrenaline attenuated TNF-α and increased IL-10 production [343, 347]. These effects are likely mediated by β_2-AR activation [346].

1.3.2.2 Cortisol

Glucocorticoids, including cortisol, are typically described as anti-inflammatory mediators with clinical uses in many acute and chronic inflammatory conditions, including asthma, Chronic Obstructive Pulmonary Disease (COPD), allergies, eczema, rheumatoid arthritis, bacterial meningitis, and Tuberculosis (TB). More recently, it has been used for patients hospitalised with Coronavirus Disease 2019 (COVID-19) who required O_2 [348]. However, some studies suggest that

glucocorticoids also exert pro-inflammatory effects. It has been suggested that the nature of the response to glucocorticoids relies on several factors, including the duration of the stimulus (acute or chronic) and the physiological state of the immune system [349].

In pathological situations, glucocorticoids may function as anti-inflammatory molecules to control the inflammatory process. They modulate inflammation at the transcription level by repressing pro-inflammatory gene expression, or through post-translational mechanisms via interactions with anti-inflammatory proteins. This involves the key inflammatory transcriptional regulators NF-κB and Activator Protein-1 (AP-1) [349-351]. The primary anti-inflammatory action of glucocorticoids is to repress a plethora of pro-inflammatory genes encoding cytokines, chemokines, cell adhesion molecules, inflammatory enzymes, and receptor signalling [350, 352-354]. Glucocorticoids also promote the anti-inflammatory differentiation of macrophages, impair phagocytosis, and can induce apoptosis of T-cells, neutrophils, basophils, and eosinophils [350, 355, 356].

In contrast, under normal physiological conditions, glucocorticoids may induce a pro-inflammatory response. While chronic exposure seems to be immunosuppressive, acute exposure has been suggested to enhance the peripheral immune response by upregulation of PRRs, and cytokine and complement factors [350, 357]. These pro-inflammatory actions in response to stress include driving the expression of Toll-Like Receptor (TLR)2, NLR family Pyrin domain containing 3 (NLRP3), the purinergic P2Y2 Receptor (P2Y2R), and potentiation of TNF-α regulated pro-inflammatory genes. Mechanisms behind these opposite effects are not well understood [349]. It has also been argued that chronic elevations can lead to resistance of the immune system, ultimately increasing the production of inflammatory cytokines [358].

1.3.2.3 Thyroid hormones

The thyroid hormones T_3 and T_4 play essential roles in both innate and adaptive immune responses. Their action is both genomic and non-genomic, and involves the thyroid nuclear receptor and PI3K-dependent Akt activation. Immune cell function, including ROS generation, chemotaxis, phagocytosis, and cytokine synthesis, is affected by both hypo- and hyperthyroidism. At physiological concentrations, thyroid hormones generally support basal immune cell functions, but effects on some functions and cell types are inconclusive [359].

Thyroid hormone effects on the innate immune system include regulation of neutrophils, NK cells, monocytes, macrophages, and DCs. They decrease neutrophil migration but increase neutrophil cell numbers, respiratory burst activity, NADPH oxidase, and myeloperoxidase, ultimately contributing to bacterial killing [87]. Thyroid effects on ROS production remain controversial [360]. While findings in neutrophils are relatively consistent, mixed effects have been found for NK cell activation and cytotoxicity. Physiological concentrations of T_3 increase NK cell activity, and INF-γ-induced regulation of NK cell activity. However, hyperthyroidism was associated with decreased NK cell activity [87].

Thyroid hormones affect the polarisation and differentiation of monocytes, with decreased differentiation into macrophages, and increased differentiation into DCs [360]. Thyroid hormones increase ROS production and phagocytosis by macrophages, but decrease polarisation into the M2 phenotype. Mixed effects have been found for bacterial killing, the production of inflammatory cytokines, and polarisation into the inflammatory M1 phenotype [87, 360, 361]. T_3 increased DC viability and effector function, and primed cytotoxic T-cells and Th17 cells [360, 362].

In adaptive immunity, hypothyroid states impair both humoral and cell-mediated immune responses. Variable effects on immune function have been found in hyperthyroid states, with either an enhanced or suppressed primary antibody

response by B-cells, and cell proliferation by B- and T-cells [363, 364]. Thyroid hormones promote antibody production and stimulate the humoral response, increase blood leukocyte numbers, T-cell mitogenesis, apoptosis, and ROS production [359, 365, 366].

1.3.2.4 Glucagon

Although most attention has focused on the role of glucagon in the regulation of metabolism, it also plays a role in the regulation of inflammation. Literature on this topic is less extensive compared to the immunomodulatory properties of other hormones, and have mainly focussed on asthma and diabetes. Nevertheless, glucagon effects are predominantly immuno-suppressive in a variety of different immune cells, including neutrophils, NK cells, leukocytes, and mast cells.

Glucagon enhanced neutrophil superoxide production both *in vitro* and *in vivo* [367], however at high concentrations, glucagon impaired ROS production [368]. Other glucagon-induced effects include inhibition of LPS-induced neutrophil accumulation in bronchoalveolar fluid, and impaired migration and adhesion [369]. These effects could contribute to diminished bacterial killing; indeed, blocking of the glucagon receptor in these experiments improved survival rates [368].

Other immune cells affected by glucagon include NK cells, T-cells, and mast cells. Glucagon inhibited NK cell activity and the activation of antigen-specific T-cell suppressors [370, 371]. Glucagon also reduced the ratio of pro- (IL-1β) and anti-inflammatory (IL-10) gene expression in monocyte THP-1 cells. This shift was prevented by blockade of the glucagon receptor [372]. A link between glucagon and IL-6 was found in obesity-related chronic low-grade inflammation [373]. Glucagon also inhibited T-cell and eosinophil accumulation, and $CD4^+$ T-cell proliferation and function. This inhibitory effect occurred via cAMP and in parallel with a reduction in

pro-inflammatory cytokines and chemokines [374]. Lastly, glucagon also inhibits mast cell number and activation [375].

1.3.2.5 Insulin

Immune cells express insulin receptors and can bind insulin [376, 377]. However, as with all other hormones, studies on the impact of insulin on immune cell function are contradictory. Although anti-inflammatory effects of insulin are well reported, recent findings implicate a more involved role for insulin in shaping the immune response during infection. Insulin may shift the immune response from an innate to adaptive response during prolonged immune activation. A recent review argued that the pro- or anti-inflammatory effects depend on the activation state of the cells [378]. The divergent roles of insulin could also be explained by (i) the transcriptional activity of the insulin receptor, (ii) ratios between specific isoforms of proteins in the insulin signalling pathway, (iii) insulin signalling crosstalk with other immunological pathways, and (iv) dose and time-dependent effects.

The anti-inflammatory properties include suppressed transcription of TLRs *in vitro* after LPS stimulation, and in patients with type 2 diabetes [379-381], and attenuated expression of pro-inflammatory cytokines *in vitro* after LPS stimulation and in animal models [379, 382-385]. These effects were partially mediated by inhibition of NF-κB and MAPK [386]. Secretion of IL-1β was inhibited by preventing assembly of the NLRP3 inflammasome [387]. Other *in vitro* and *in vivo* immunosuppressive effects of insulin include reduced ROS production and NET formation by neutrophils, Th2 skewing effects, decreased apoptosis of LPS-stimulated macrophages, and antagonism of the clotting cascade [379, 382, 388-390].

These anti-inflammatory effects of insulin could be mediated via distinct pathways. The first possible mechanism is lowering of glucose levels, thereby preventing glucose toxicity and cell stress, Receptor for Advanced Glycation Endproducts

(RAGE) formation, and production of ROS [378]. Secondly, insulin can antagonise activity of the FOXO1 transcription factor via activation of the P13K-Akt signalling pathway [391]. FOXO1 plays a role in phagocytosis, chemotaxis, anti-bacterial function of neutrophils, transcription of pro-inflammatory genes by macrophages, polarisation of macrophages into the M1 subtype, and also impacts on adaptive immunity [391-393]. Other possible pathways are: indirect regulation of the transcription factor NRF2, which plays a role in regulating antioxidant defences [378]; suppression of transcriptional activity of NF-κB; and modulation of autophagy [379, 394, 395].

As mentioned earlier, insulin exhibits pro-inflammatory properties on lymphocytes, NK cells, and other innate immune cells. Insulin plays a critical role in promoting effector function, proliferation, and differentiation of T-cells via increased aerobic glycolysis, glucose uptake, and activation of the anabolic P13K-Akt-mTOR pathway [396, 397]. T-cells showed upregulation of insulin receptors upon activation [398]. Via this same signalling pathway, hyperinsulinaemia compromised Treg function to secrete IL-10, but elevated expression of INF-γ in obese mice [399]. Despite these effects on T-cells, insulin showed no effects on antibody production [400]. Insulin also promoted NK cell function by increasing the expression of INF-γ and cytotoxicity [401]. Hypoinsulinaemia in these same cells or suppression of insulin receptors promoted death.

Considering innate immune cells, insulin potentiated LPS-induced pro-inflammatory cytokine release in bone marrow derived macrophages, and in alveolar and peritoneal macrophages from diabetic mice [385, 402]. Similar increases in LPS-induced pro-inflammatory cytokine secretion, ROS production, and Methylcyclopropene (MCP)-1 directed migration have been reported in human monocytes and macrophages [403-405]. This pro-inflammatory pattern was mediated by the NLRP3 inflammasome [402]. Deficiency of the insulin receptor on macrophages reduced proliferation and

migration, and shifted macrophage polarisation from a M1 to M2 subtype [406, 407]. Insulin also promoted macrophage survival, although the consequences for the immune system are unknown [408].

Interestingly, insulin suppressed ROS production by macrophages, but increased ROS in neutrophils. Although it remains unclear why these cells are differentially regulated, underlying mechanisms might include increased formation of NADPH by glucose loading and promoting activity of NADPH Oxidase 2 (NOX2) [409]. In another study, neutrophils did not induce a respiratory burst, but primed neutrophils for a more robust ROS production following a challenge [410]. Lastly, insulin steered differentiation of bone marrow progenitor cells towards a lymphoid cell lineage [411]. Despite this, increased circulating levels of leukocytes by insulin seems to be driven by an increase in neutrophils, potentially due to suppressed expression of adhesion molecules and a subsequent increased release from the bone marrow [412].

1.3.3 Endocrine therapy during sepsis

International guidelines on the appropriate management of sepsis and septic shock have recently been revised [8]. Both hyperglycaemia and hypoglycaemia are associated with increased mortality in critically ill patients [413-415]. It is therefore *strongly* recommended to initiate insulin therapy in adult patients with sepsis at a glucose level of ≥180mg/dL, aiming for a blood glucose range of 144-180 mg/dL (moderate quality evidence).

To restore blood circulation and tissue perfusion in adult patients with septic shock, noradrenaline is *strongly* recommended as the first-choice vasopressor agent. When noradrenaline use cannot achieve an adequate blood pressure goal, it is *weakly* suggested to add vasopressin as a second agent (moderate quality evidence). Only when the combination of these two still results in inadequate restoration of blood pressure is the addition of adrenaline *weakly* recommended (low quality evidence).

These guidelines change for adult patients with septic shock and cardiac dysfunction with persistent hypoperfusion, despite an adequate volume status and arterial blood pressure. In such cases, it is *weakly* suggested to add either dobutamine to noradrenaline, or to solely use adrenaline (low quality evidence).

The guidelines advise against the use of corticosteroids to treat septic shock patients if adequate fluid resuscitation and vasopressor therapy can restore haemodynamic stability. The use of corticosteroids, and specifically hydrocortisone, is only *weakly* recommended for adult patients with septic shock and an ongoing requirement for vasopressor therapy (noradrenaline/adrenaline use ≥0.25 mcg/kg/min for at least 4 hours). As evidence on the optimal dosing regimen remains inconclusive, the guidelines suggest initiating hydrocortisone at a dose of 200 mg/day, either as a continuous infusion or as a 50 mg bolus every 6 hours (moderate quality of evidence).

1.4 Summary

Sepsis is a syndrome defined as a severe dysregulated response of the body to infection, leading to multiple organ dysfunction requiring drug and device support [1]. The initial recognition of a pathogen by the immune system leads to a systemic inflammatory response, and subsequent activation of a range of complex and biphasic interactions. These involve multiple pathways including autonomic, cardiovascular, endocrine, metabolic, bioenergetic, and immune systems [14]. Although the precise pathophysiology of MOF during sepsis remains unclear, it appears to relate to metabolic dysfunction rather than structural damage [104-106]. A metabolic shutdown, driven by a lack of sufficient ATP generated by the mitochondria, could well explain this phenomenon [107].

Decreased mitochondrial activity and a metabolic shutdown could potentially also explain immunosuppression seen with prolonged sepsis. During the first few days following infection, a hyper-inflammatory response dominates the clinical picture.

However, after this initial activation, circulating immune cells are downregulated, leading to immune suppression [18, 19]. Alterations in mitochondrial respiration of monocytes and lymphocytes are associated with sepsis severity, functional immune suppression, and an increased risk of mortality (both short- and long-term) [135, 145, 146]. This state of metabolic dormancy could be induced via inflammatory cytokine- and hormone-mediated effects on cellular energy production [176].

Hormones likely play a crucial but underappreciated role during sepsis, with the degree of abnormality reflecting an increased risk of death [61, 62]. A typical biphasic response can be observed in septic patients; an initial acute rise in stress hormones is seen, which aims to maintain an adequate circulation and tissue oxygenation, and increase metabolic activity and ATP synthesis [416]. There is a concurrent shutdown of less vital systems and anabolism is inhibited. Another hallmark of sepsis is the induction of insulin resistance, where normal concentrations of insulin produce a subnormal response [58]. The hormonal profile also alters substantially with the onset of the ‘sick euthyroid syndrome’, and reduced adrenal responsiveness to ACTH, often despite hypercortisolaemia [60, 416].

In addition to the endogenously released hormones as part of the host response to sepsis, insulin, catecholamines such as noradrenaline, and steroid hormones including hydrocortisone, are often administered in high concentrations as part of sepsis therapy. The magnitude of changes in endogenous levels and these exogenously administered hormones could significantly contribute to decreased mitochondrial activity, a metabolic shutdown, and in turn, functional disturbances of organ systems including immune cells.

1.5 Aims and hypothesis

It is hypothesised that temporal changes in endocrine levels during sepsis modify mitochondrial function of mononuclear immune cells, contributing to alterations in

effector function, ultimately leading to worse outcomes in patients. By intervening at the right time and with the right dose, mitochondrial function can be restored, and immune cell function improved.

To study the effects of changes in stress and metabolic hormones during sepsis on mononuclear immune cell mitochondrial and effector function, a variety of models and techniques will be used, including samples from septic patients, *in vitro* models with isolated primary immune cells, and an *in vivo* rat model of faecal peritonitis. More specifically:

1) The endocrine, mitochondrial, mononuclear immune, and inflammatory response in human sepsis will be characterised, to see if there are any correlations between hormone levels, mitochondrial and immune function.

2) The impact of a selection of stress hormones, namely catecholamines and cortisol, in various concentrations will be studied on mitochondrial and mononuclear immune cell function in an *in vitro* model of infection with isolated primary cells.

3) The temporal endocrine and inflammatory response in a long-term (3-day), fluid resuscitated rat model of faecal peritonitis for future *in vivo* studies will be characterised.

Chapter 2 Endocrine, immune, and mitochondrial changes in sepsis; an observational study

2.1 Introduction

Temporal changes in levels of stress and metabolic hormones during sepsis, and their correlation with mitochondrial and monocyte function were studied at the ATTIKON University Hospital in Athens, Greece. This collaboration with Prof. Giamarellos-Bourboulis was part of the Horizon2020 Marie-Skłodowska-Curie ESA. Mitochondrial and immune function of monocytes were measured by flow cytometry analysis of fresh whole blood collected after hospital admission on Days 1, 2, 4 and 7. Serum was collected from the same patients to assess levels of inflammatory markers and various stress and metabolic hormones, including catecholamines (adrenaline and noradrenaline), cortisol, thyroid hormones (fT_3 and rT_3), insulin, and glucagon.

These hormones were specifically selected specifically after a detailed literature search. Changes in levels of these hormones during both the acute and prolonged phases of sepsis have been associated with worse outcomes in sepsis, and clear effects on mitochondrial and immunomodulatory properties have been reported in the literature. Biphasic changes in these hormones along with their effects on mitochondrial and immune cell function have been discussed in Chapter 1.

2.2 Methodology

2.2.1 Study design

This study was a prospective, observational cohort study of patients with sepsis admitted to the ATTIKON University Hospital, KAT Attica General Hospital, and Alexandra General Hospital in Athens, Greece. The Hellenic Institute for the Study of

Sepsis has been running an ongoing observational study in 65 hospitals in Greece since 2006, during which blood samples and patient data are collected from septic patients. The current study made use of blood samples already being collected as part of this ongoing study in three hospitals (ATTIKON University Hospital, KAT Attica General Hospital, and Alexandra General Hospital), located in Athens. This ongoing study was reviewed and approved by the Ethics Committees of the participating sites (Appendix I). Additional ethics approval was received from UCL's ethics committee (No. 16347/001). A material data transfer agreement was also put in place between the two institutions.

Following hospital admission and enrolment into the study, no investigational treatments were undertaken, and patients therefore received usual care. A total of 18 mL of blood was withdrawn from pre-inserted arterial lines the morning after hospital admission (Day 1), and then repeated on Days 2, 4 and 7. All samples were collected between the hours of 08:00 and 09:00 each morning to reduce the effects of diurnal variation, although this is often lost in sepsis. In patients who did not have a patent arterial line, blood was aspirated from a central venous catheter if present, or by venepuncture.

Blood was collected into commercially available Blood Collection Tubes (BCTs). These consisted of two 4 mL sodium heparin tubes (BD Vacutainer® Green, BD Diagnostics, Franklin Lakes, USA) and one 10 mL serum tube containing silica to accelerate coagulation time for serum separation (BD Vacutainer® Red, BD Diagnostics). Samples were transferred immediately to the central lab for analyses. Serum tubes were centrifuged at 3500 Revolutions Per Minute (RPM) for 10 minutes, supernatant was decanted into 500 uL aliquots and stored at -80°C. Whole blood in sodium heparin tubes was used for flow cytometry analysis of monocyte mitochondrial and effector function.

2.2.2 Study population

The emergency departments of hospitals in Athens rotate their shifts so that every hospital is responsible for emergency admissions once every 8 days. Adult patients admitted to these emergency departments from May-July 2018 were screened for in- and exclusion criteria upon admission. In addition, patients admitted to the ICU were screened for the same criteria daily (see below for overview). Patients with mental competency were approached directly for consent. However, in most cases, patients lacked the capacity to consent. In these cases, nominee approval from a patient's next-of-kin was sought out, followed by patient consent once capacity had been regained. A group of control patients undergoing elective surgery at the ATTIKON University Hospital was also included in the current study. Patients with metastatic cancer were excluded. Participant information sheets and consent forms are reproduced in Appendix III (in Greek).

A formal sample size calculation was not performed, but a sample size of 30 septic patients was selected based on previous studies assessing temporal endocrine changes and inflammatory markers during sepsis (Studying Responses of the Stress System (STRESS) study at UCL Hospital (UCLH)) [417].

Patient demographics were recorded upon admission to the hospital. This information included patient's age, sex, and underlying disease or co-morbidities. Information on patient status, including the Acute Physiology and Chronic Health Evaluation II (APACHE II) illness severity score [418], Sequential Organ Failure Assessment (SOFA) score [419], and the Charlson's Comorbidity Index (CCI) [420] were calculated upon admission, with subsequent calculation of the SOFA score coinciding with the days. Physiological, haematological, and biochemical measurements were recorded in addition to information on medication, source of infection, and outcome.

Inclusion criteria

- Adult patients (≥18 years)
- Patients of both sexes
- Informed Consent Form (ICF) signed by patient or by first-degree relative if the patient lacks competency to give consent
- Sepsis defined by the Sepsis-3 definitions (acute change in SOFA score ≥2 related to infection)

Exclusion criteria

- Paediatric patients (<18 years)
- Pregnant or lactating patients
- Patients who have used cortisol-altering drugs within the 6 weeks prior to admission (e.g. steroids, with the exception of hydrocortisone for septic shock)
- Cirrhosis (Child Pugh B or C)
- Severe psychiatric illness
- Severe brain injury (e.g. trauma, stroke, prolonged cardiac arrest)
- Any stage IV malignancy
- Active TB as defined by the co-administration of drugs for the treatment of TB
- Infection by the human immunodeficiency virus (HIV)
- Any primary immunodeficiency
- Any anti-cytokine biological treatment within the past month
- Medical history of systemic lupus erythematosus (SLE)
- Medical history of Multiple Sclerosis (MS) or any other demyelinating disorder

2.2.3 Flow cytometry

2.2.3.1 Staining protocols

Flow cytometry was performed with the Cytomic FC500 flow cytometer (Beckman Coulter, Marseille, France) using fresh whole blood collected in sodium heparin BCT. From this tube, 50 uL was transferred into 5 mL round-bottom polystyrene tubes (Sarstedt, Nümbrecht, Germany). Whole blood was lysed with 500 uL 1x VersaLyse Lysing Solution in De-ionised H_2O (dH_2O) (Beckman Coulter) and incubated at room

temperature for 15 minutes to eliminate interfering erythrocytes. Cells were washed with 4 mL of Phosphate Buffered Saline (PBS) (Biowest, Nuallé, France) and spun down at 1200 RPM for 5 minutes. After washing, the PBS was discarded, and four different staining protocols were initiated (Table 1). Annexin-V-FITC (Beckman Coulter) and 7AAD (Beckman Coulter) were used as markers of apoptosis and necrosis in all protocols. Anti-CD14-PC7 (Beckman Coulter) was used to identify monocytes, as CD14 antigen is present on most peripheral blood monocytes [421].

The first staining protocol was used to measure MMP by Tetramethyl-Rhodamine Methyl Ester (TMRM) (Thermo Fisher Scientific, Waltham, MA, USA). This mitochondria-selective cationic dye accumulates in the mitochondrial matrix based on charge; depolarisation of the IMM allows less TMRM to accumulate. Hence, if the IMM permeability increases, the signal for TMRM will reduce. Cells were incubated for 30 minutes with a final TMRM concentration of 125 nM in a dark 5% CO_2 incubator at 37°C.

After incubation, 50 uL of 1x Annexin Binding buffer (Beckman Coulter), 5 uL Annexin V-FITC, 10 uL 7AAD, and 5 uL of anti-CD14-PC7 were added. Cells were incubated at room temperature for 15 minutes in the dark. 400 uL 1x Annexin Binding buffer was added at last before analysis. Carbonyl Cyanide-P-(Trifluoromethoxy) Phenylhydrazone (FCCP), an uncoupler of the ETC, was used as a control for TMRM in a separate fully stained sample. In that case, cells were first incubated with FCCP at a final concentration of 50 uM for 15 minutes at 37°C with 5% CO_2.

A second protocol was used to measure mROS production in these cells. Cells were incubated with MitoSox™ Red (Invitrogen, Eugene, OR, USA) at a final concentration of 2 uM for 15 minutes in a dark 37°C incubator with 5% CO_2. After incubation, Annexin Binding buffer, Annexin V-FITC, 7AAD, and anti-CD14-PC7 were added, as described previously. As a positive control for MitoSox, cells were incubated with antimycin A (Sigma-Aldrich, St Louis, MI, USA) at a concentration of 100 uM in a

separate fully stained sample. Antimycin A, an inhibitor or respiratory Complex III, was added after MitoSox to induce mROS production. For this, cells were incubated for 15 minutes at 37°C with 5% CO_2.

HLA-DR receptor expression was measured by anti-HLA-DR,DP,DQ-PE antibody (Beckman Coulter). This antibody recognises human class II MHC antigens [422, 423]. It was added in 5 uL to the lysed cells together with 1x Annexin Binding buffer, Annexin V-FITC, 7AAD and anti-CD14-PC7, as described previously. The isotype control IgG1 mouse-PE (Beckman Coulter) was used to exclude nonspecific binding. For simplicity, this antibody and subsequent results will be referred to as (anti-)HLA-DR in this thesis.

Table 1. Flow cytometry panels for MitoSox, TMRM, and HLA-DR.

			MitoSox panel		**TMRM panel**		**HLA-DR panel**	
Laser	**Filter**	**Fluorochrome**	**Target**	**Clone**	**Target**	**Clone**	**Target**	**Clone**
Argon (488 nm)	525/40	FITC	Annexin-V		Annexin-V		Annexin-V	
	575/40	PE	MitoSox		TMRM		HLA-DR	9-49
	675/40		7AAD		7AAD		7AAD	
	755/40	PC7	CD14	RMO052	CD14	RMO052	CD14	RMO052

For quantification of HLA-DR signal to receptor expression per cell, Quantibrite™ anti-human HLA-DR PE/Monocyte PerCp-Cy5.5 was used (BD Biosciences, San Jose, CA, USA). A total of 10 uL Quantibrite was added to 25 uL whole blood, and incubated at room temperature in the dark for 30 minutes. Subsequently, 250 uL of 1x Versalyse in dH_2O (Beckman Coulter) was added and incubated at room temperature in the dark for another 15 minutes. After incubation, 3 mL of PBS was added, and cells were spun at 1200 RPM for 5 minutes. The PBS was discarded and cells were resuspended in 500 uL of IOTest3 Fixative Solution (Beckman Coulter) with 3% formaldehyde in PBS. Quantibrite-PE calibration beads (BD Biosciences) were run monthly for conversion of the Mean Fluorescence Intensity (MFI) signal into receptor expression per cell.

In conjunction with the above stained samples, compensation was set up before each experiment using single stained control samples. To induce maximum fluorescence, single stained samples for 7AAD and Annexin-V were first heat killed (60°C for 15 minutes). Unstained control samples were used for each patient separately. Fluorescence Minus One (FMO) controls were used to set gating strategies. Quality control was performed daily using Rainbow Calibration Particles (BD Biosciences). Data obtained by flow cytometry were analysed using FlowJo software Version 10 (Tree Star Inc., Ashland, OR, USA).

2.2.3.2 Acquisition and gating strategy

Cell populations were identified using sequential gating strategies in FlowJo Version 10 (Tree Star Inc.) (Figure 3). Firstly, using a Sideward Scatter (SSC) and Forward Scatter (FSC), cells were distinguished from debris. From these cells, monocytes were identified based on SSC and expression of CD14. This cell population was subsequently gated on a 7AAD and Annexin-V plot, enabling live, dead, and apoptotic cells to be distinguished from each other. The geometric mean for TMRM, MitoSox, and HLA-DR was subsequently calculated for $7AAD^-$ (live) $CD14^+$ monocytes. A total of 2,500 $CD14^+$ monocytes were acquired for each patient by flow cytometry.

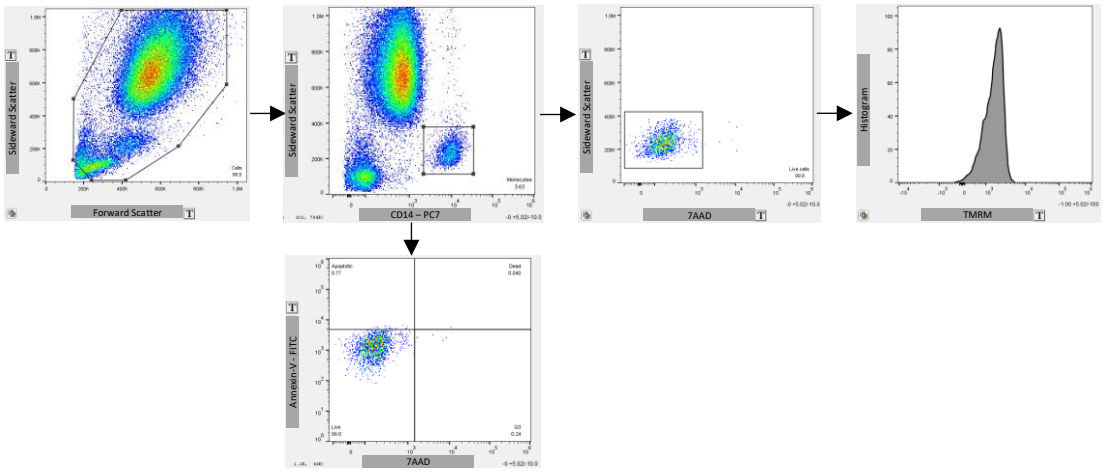

Figure 3. Gating strategy for TMRM, MitoSox, and HLA-DR.

For Quantibrite HLA-DR, a separate gating strategy was used (Figure 4). Again, using a SSC and FSC plot, cells were separated from debris. From these cells, $CD14^+$ subsets were identified and the geometric mean for HLA-DR in this population calculated. A total of 2,500 $CD14^+$ monocytes were acquired for Quantibrite HLA-DR. Using monthly run Quantibrite PE beads with known mean PE molecules/bead, the MFI for HLA-DR could then be converted into receptor expression per cell (mAB/cell).

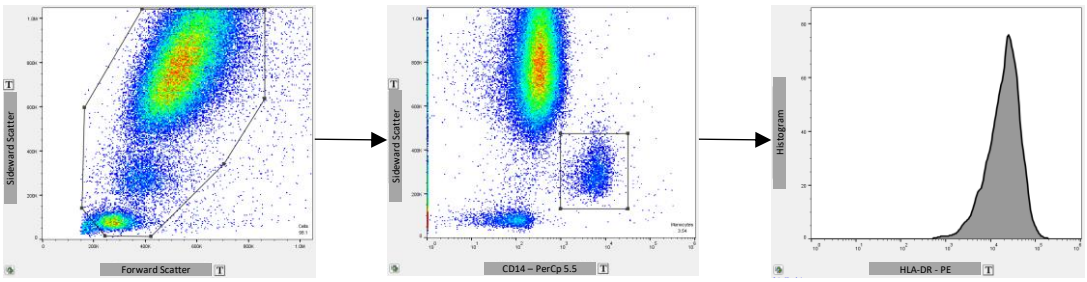

Figure 4. Gating strategy for Quantibrite HLA-DR.

2.2.4 Biochemical analyses

To assess the endocrine response during sepsis, multiple biochemical Enzyme-Linked Immunosorbent Assays (ELISA) were performed with patient serum samples collected on multiple days. Six samples of serum from both survivors, non-survivors, and control patients were tested in three different dilutions. The dilution providing the most accurate detection for each analyte was subsequently used for further analyses. A LEGENDplex™ Multiplex Assay (BioLegend, San Diego, CA, USA) was used to measure cytokines and chemokines. All ELISAs and multiplex assays were performed in duplicate and following the manufacturer's instructions.

For measurement of catecholamines (noradrenaline and adrenaline), thyroid hormones (fT_3 and rT_3), and glucagon, competitive ELISA kits were used (Elabscience Biotechnology Co., Beijing, China). Both catecholamines were analysed in a 1:5 dilution, thyroid hormones in a 1:2 dilution, and glucagon in a 1:10 dilution. Insulin was measured using a human insulin ELISA (EZHI-14K, Sigma-Aldrich) with neat serum

samples. For cortisol, a 1:5 dilution was used (ab108665, Abcam, Cambridge, UK). Although this last ELISA kit did not elaborate on its specificity for hydrocortisone, its specificity for cortisone was only 3.69%. Signal quantification of the 96-well hormone ELISAs was measured using a Synergy 2 spectrophotometric plate reader (Biotek, Winooski, VT, USA) set to the appropriate wavelength according to manufacturer's instructions. Origin 2019 software (OriginLab Corp., Northampton, MA, USA) was used to produce a four-parameter logistic curve from the Optical Density (OD) values of the reference standards for the hormone ELISAs, allowing calculation of sample analyte concentrations.

LEGENDplex HU Essential Immune Response Panel (13-plex) w/VbP (BioLegend) was used to measure multiple cytokines and chemokines, including IL-4, IL-2, IP-10, IL-1β, TNF-α, MCP-1, IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, IL-8, and Transforming Growth Factor (TGF)-β1. Serum samples were analysed in a 1:2 dilution according to the manufacturer's recommendations, and measured by flow cytometry (FACS Verse, BD Biosciences). These measurements were then analysed using BioLegend's LEGENDplex data analysis software.

2.2.5 Statistical analysis

Microsoft Office Excel (Microsoft, Redmond, WA, USA) was used to organise and summarise data, before statistical analysis and data visualisation with GraphPad Prism Version 9 (GraphPad Software, San Diego, CA, USA). Normality was assessed by inspection of QQ plots and Shapiro-Wilk test for normality, with parametric or non-parametric tests used accordingly. For comparison of the three groups at Day 1, one-way Analysis of Variance (ANOVA) with Fisher's Least Significant Difference (LSD) multiple comparisons, or the non-parametric Kruskal-Wallis test with Dunn's multiple comparisons, were used. I did not correct for multiple comparisons due to low patient numbers. For binominal data, the Chi-square test was used. Pearson correlation

coefficients were calculated for correlation between parameters and visualised in correlation matrixes. A *P*-value <0.05 was considered significant.

2.3 Results

2.3.1 Patient recruitment

As shown in Figure 5, 24 patients were initially eligible for inclusion in the current study. These patients were recruited at three different hospitals in Athens, with the majority enrolled at the ATTIKON University Hospital (*n*=20), two at Alexandra General Hospital, and one at the KAT Attica General Hospital.

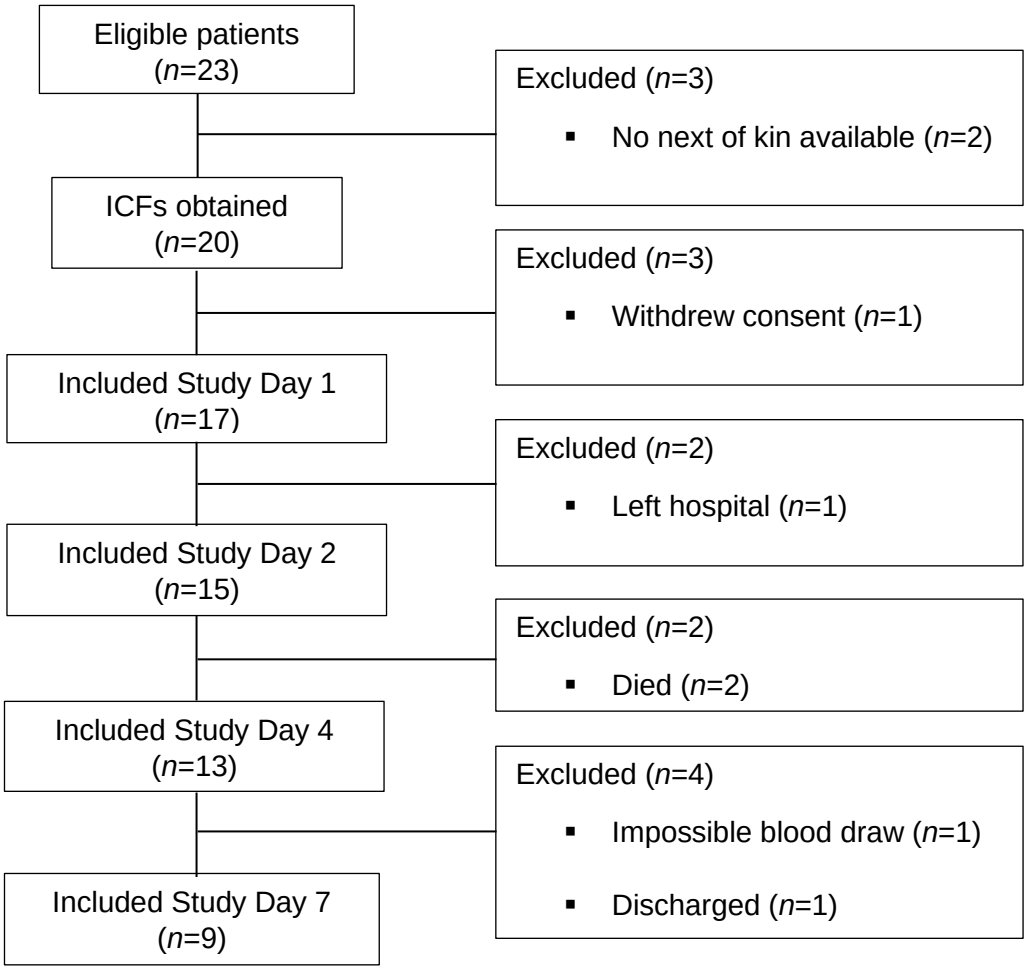

Figure 5. Flow chart of recruitment and inclusion of patients with sepsis. Patients with sepsis were subsequently divided into survivors and non-survivors based on 28-day mortality. ICF: Informed Consent Form.

Of the 23 eligible patients, 20 consented to take part in the study. However, before the first blood sampling, two patients passed away and one family withdrew consent, leaving 17 patients for study enrolment. During the consecutive days, some patients left the hospital voluntarily (*n*=1), were discharged (*n*=1), died (*n*=5), or samples could not be collected because of impossible blood draw (*n*=1), leaving 9 patients with samples taken on all days.

All control patients undergoing elective surgery were recruited from the ATTIKON University Hospital (*n*=13). These patients either underwent laparoscopic cholecystectomy (*n*=4), hepatectomy (*n*=2), appendectomy (*n*=2), removal of a perianal abscess (*n*=2), gastrectomy and splenectomy (*n*=1) or penectomy (*n*=1).

2.3.2 Patient demographics and characteristics

Demographic data were recorded for pre-operative controls and septic patients. In addition to basic demographic data, information was collected on diagnosis, comorbidities, and treatment. The CCI, APACHE II, and SOFA scores on Day 1 were calculated (Biochemical analyses of blood on Day 1 was performed by the hospital laboratory and subsequently retrieved from medical records. No significant differences were found between sepsis survivors and non-survivors for any of these parameters. No measurements were available for the pre-operative control patients, but normal ranges as found in the literature are indicated in Supplementary Table 1.

The Partial Pressure of O2 (pO2) was higher in non-survivors compared to the normal range. The same applies to glucose, indicating hyperglycaemia, and creatinine, indicating kidney failure in these patients. White blood counts (WBCs) were higher in all septic patients compared to the normal range. Low bicarbonate (HCO3-) levels were found in the blood of septic non-survivors, compared to the normal range. A low ratio of pO2 to fractional inspired O2 (pO2/FiO2), and low levels of partial pressure of

CO2 (pCO2), and haemoglobin (Hb) were found in all septic patients when compared to the normal range.

). Based on 28-day survival, septic patients were separated into survivors and non-survivors for subsequent analysis. The overall 28-day mortality rate was 41.2%, with all deaths occurring within the first 11 days of admission (Figure 6).

When comparing basic demographics, non-surviving septic patients were generally older than pre-operative control patients (ANOVA: *P*=0.04; Fisher's LSD: *P*=0.02). Higher severity scores were found in septic patients compared to controls, with significantly higher APACHE II scores in sepsis non-survivors compared to survivors (*P*=0.02). Most patients with sepsis were diagnosed with pneumonia, followed by urinary tract infections, acute cholecystitis, primary bacteraemia, and gastroenteritis.

Table 2. Patient demographics and characteristics on Day 1. Data from patients with sepsis (divided into survivors (SS) and non-survivors (SNS) and pre-operative control patients was collected on the first day after admission. Data presented as mean ± SD unless indicated otherwise. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor.

	Control (*n*=13)	**SS (*n*=10)**	**SNS (*n*=7)**	***P*-value**
Demographics				
Age - year	67 ± 21	78 ± 10	87 ± 5	0.04
Male sex – no./total no. (%)	7/13 (53.8)	6/10 (60.0)	2/7 (28.6)	0.41
Site of infection – no./total (%)				
Pneumonia		5/10 (50.0)	4/7 (57.1)	
Urinary tract		2/10 (20.0)	2/7 (28.6)	
Acute cholecystitis		2/10 (20.0)		
Primary bacteraemia			1/7 (14.3)	
Gastroenteritis		1/10 (10.0)		
Clinical data				
CCI score (maximum 24)	4 ± 3	7 ± 3	7 ± 2	0.11
APACHE II score		14 ± 6	25 ± 8	0.02

SOFA score (maximum 24)		4 ± 2	8 ± 4	0.08
Co-morbidities – no./total (%)				
Diabetes	4/13 (30.8)	5/10 (50.0)	1/7 (14.3)	
Chronic kidney disease		1/10 (10.0)	1/7 (14.3)	
Chronic renal failure		2/10 (20.0)		
Heart failure		3/10 (30.0)	1/7 (14.3)	
Arterial hypertension	8/13 (61.5)	1/10 (10.0)		
Coronary heart disease			1/7 (14.3)	
Atrial fibrillation	1/13 (7.7)		3/7 (42.9)	
Cerebrovascular disease			1/7 (14.3)	
Cerebral ischaemic attack		1/10 (10.0)	1/7 (14.3)	
Cerebral haemorrhage			1/7 (14.3)	
COPD		1/10 (10.0)		
Hypothyroidism			1/7 (14.3)	
Dyslipidaemia	2/13 (15.4)			
Gallstones		1/10 (10.0)		
Dementia		3/10 (30.0)	2/7 (28.6)	
Medication				
Noradrenaline – no. patients (%)			3/7 (28.6)	
Noradrenaline – dose mg/day			60.2 ± 73.58	
Hydrocortisone –no. patients (%)		1/10 (10.0)	2/7 (28.6)	
Hydrocortisone – dose mg/day		150	200 ± 0	

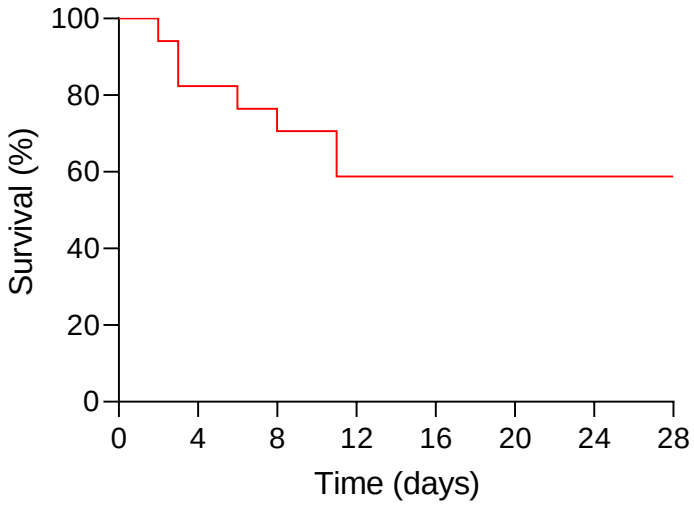

Figure 6. Kaplan-Meier 28-day survival curve for patients with sepsis (*n*=17). In these patients, the 28-day mortality rate was 41.2%.

Although only 3/7 (42.9%) of sepsis non-survivors received noradrenaline on Day 1, this increased to 5/7 (71.4%) patients on the following days (data not shown). For hydrocortisone administration, the number of sepsis non-survivors receiving this treatment increased from 2/7 (28.6%) to 3/7 (42.9%) over subsequent days. To our knowledge, no patients received any other type of endocrine therapy.

Biochemical analyses of blood on Day 1 was performed by the hospital laboratory and subsequently retrieved from medical records. No significant differences were found between sepsis survivors and non-survivors for any of these parameters. No measurements were available for the pre-operative control patients, but normal ranges as found in the literature are indicated in Supplementary Table 1.

The Partial Pressure of O_2 (pO_2) was higher in non-survivors compared to the normal range. The same applies to glucose, indicating hyperglycaemia, and creatinine, indicating kidney failure in these patients. White blood counts (WBCs) were higher in all septic patients compared to the normal range. Low bicarbonate (HCO_3^-) levels were found in the blood of septic non-survivors, compared to the normal range. A low ratio of pO_2 to fractional inspired O_2 (pO_2/FiO_2), and low levels of partial pressure of CO_2 (pCO_2), and haemoglobin (Hb) were found in all septic patients when compared to the normal range.

2.3.3 Biochemical analyses

2.3.3.1 Hormone measurements

ELISA assays were performed with patient serum samples that had been stored at -80°C. Samples were taken on Day 1, 2, 4 and 7 for septic patients (survivors and non-survivors), and at one timepoint for pre-operative control patients. As the number of patients decreased on consecutive days due to deaths and discharge, results for all groups were analysed and visualised for Day 1 only. Some of the patients included

received endocrine therapy, either noradrenaline or hydrocortisone, as part of their treatment. These patients are indicated in the graphs below.

For noradrenaline and adrenaline, differences between all groups did not reach significance (*P*=0.34 and *P*=0.98, respectively) (Figure 7). However, adrenaline in many of the patient samples fell below the detectable limit. As there is no biological rationale to explain this, it suggests a methodological issue with the assay, despite excellent standard curves being obtained.

Significant differences were found for cortisol, with higher levels in sepsis non-survivors compared to pre-operative control patients (ANOVA: *P*=0.04; Fisher's LSD: *P*=0.01). The difference between pre-operative control patients and sepsis survivors almost reached significance (*P*=0.09).

For both thyroid hormones, no differences were found between groups (*P*=0.56 for fT_3, and *P*=0.97 for rT_3) (Figure 8). Looking at the ratio of fT_3/rT_3, there was a trend towards a reduced ratio in septic non-survivors compared to control patients (ANOVA: *P*=0.13; Dunn's: *P*=0.04).

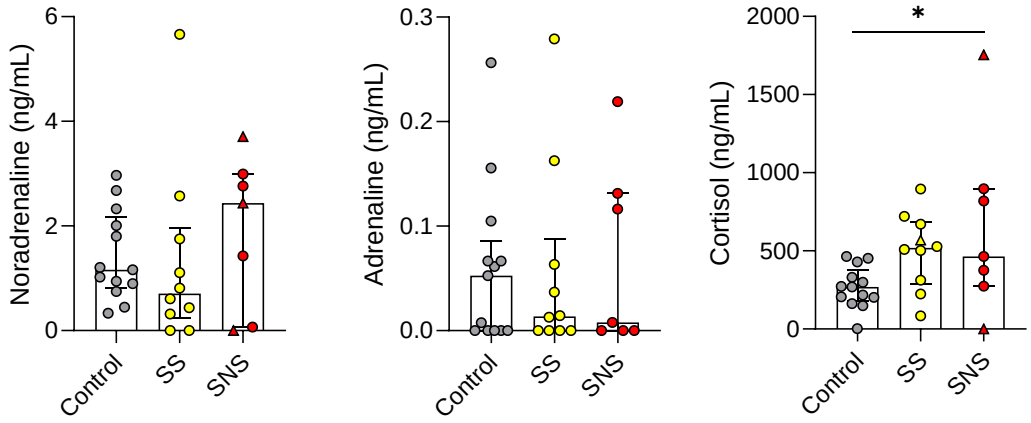

Figure 7. Serum levels of noradrenaline, adrenaline, and cortisol on Day 1. Hormones were measured by ELISA in serum samples from patients with sepsis (divided into survivors (SS) and non-survivors (SNS)) and pre-operative control patients on the 1st day after admission. Data presented as median ± IQR for noradrenaline and adrenaline, and as mean ± SD for cortisol. ▲: patient receiving either noradrenaline or hydrocortisone. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor. *P<0.05.

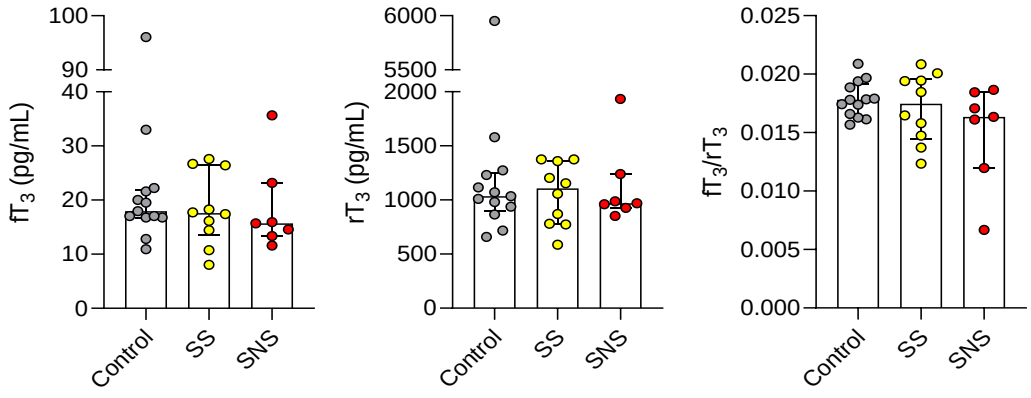

Figure 8. Serum levels of fT_3 and rT_3 on Day 1. Thyroid hormones were measured by ELISA in serum samples from patients with sepsis (divided into survivors (SS) and non-survivors (SNS)) and pre-operative control patients on the 1st day after admission. Data presented as median ± IQR for fT_3 and rT_3, and mean ± SD for the ratio of fT_3/rT_3. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor.

No significant differences in serum levels of insulin were found between groups (*P*=0.50). Some sepsis non-survivors had very high insulin levels. Similar results were obtained for glucagon (*P*=0.21) (Figure 9).

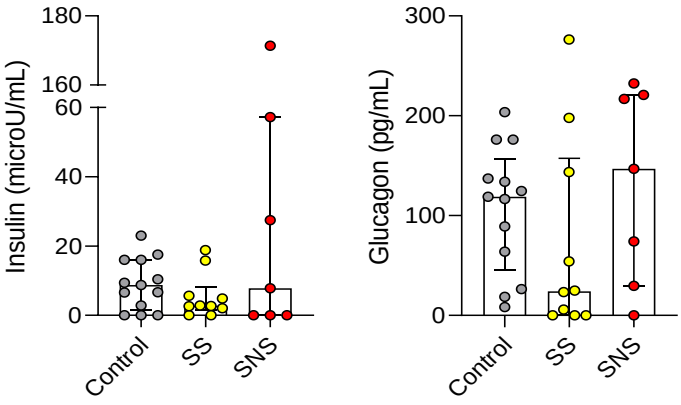

Figure 9. Serum levels of insulin and glucagon on Day 1. Hormones were measured by ELISA in serum samples from patients with sepsis (divided into survivors (SS) and non-survivors (SNS)) and pre-operative control patients on the 1st day after admission. Data presented as median ± IQR for all. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor.

2.3.3.2 Multiplex cytokines and chemokines

Serum cytokines and chemokines were measured using a 13-panel multiplex assay. Levels of these parameters for Day 1 only were analysed and visualised in graphs, due to the low number of patients on subsequent days. One pre-operative control sample was lost and could therefore not be included in this analysis.

Levels of the anti-inflammatory cytokines IL-4, IL-10, and TGF-β1 did not reach significance between groups (*P*=0.99, *P*=0.98, and *P*=0.46 respectively) (Figure 11). The concentration of most generally pro-inflammatory cytokines, including IL-2, IL-1β, TNF-α, IL-17A, and IL-12p70 was higher in septic patients, especially in non-survivors, but there was no statistical significance between groups (*P*=0.30, *P*=0.71, *P*=0.62, *P*=0.55, and *P*=0.64, respectively) (Figure 10). A similar trend for IL-6 was found in sepsis survivors compared to pre-operative control patients (ANOVA:

P=0.06; Fisher's LSD: *P*=0.03). The difference in IL-6 between sepsis non-survivors and pre-operative control patients almost reached significance (*P*=0.08).

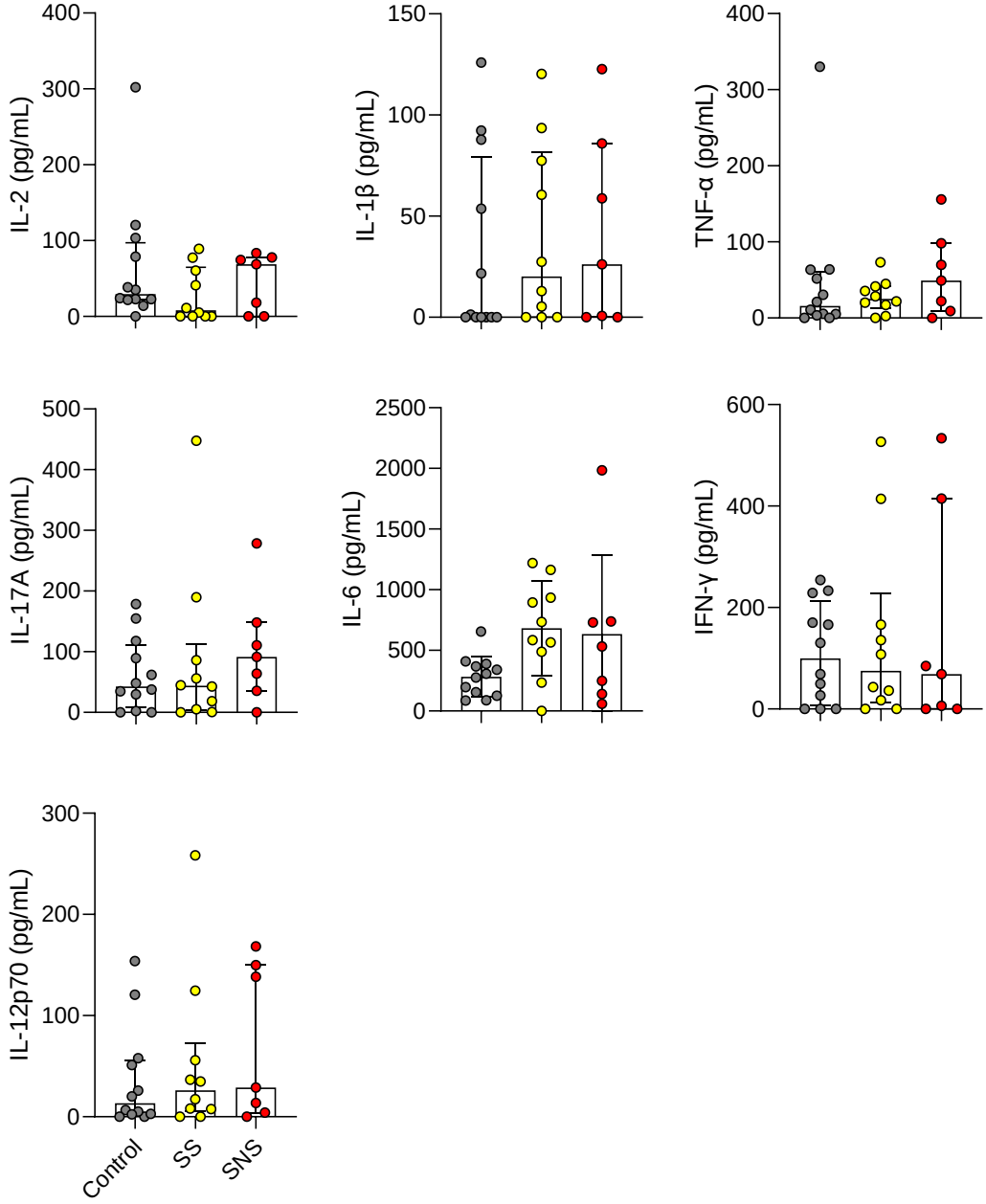

Figure 10. Serum levels of pro-inflammatory cytokines on Day 1. Pro-inflammatory cytokines were measured by multiplex assay in serum samples from patients with sepsis (divided into survivors (SS) and non-survivors (SNS)) and pre-operative control patients on the 1st day after admission. Data presented as median ± IQR for all cytokines, apart from IL-6 where data are presented as mean ± SD. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor.

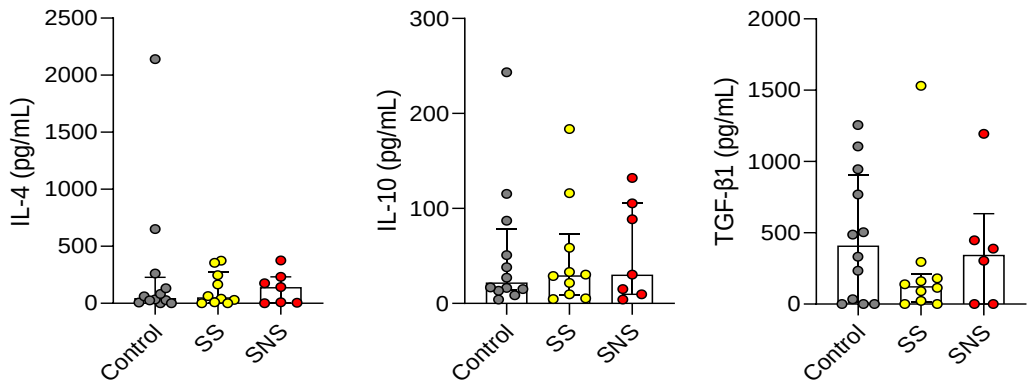

Figure 11. Serum levels of anti-inflammatory cytokines on Day 1. Anti-inflammatory cytokines were measured by multiplex assay in serum samples from patients with sepsis (divided into survivors (SS) and non-survivors (SNS)) and pre-operative control patients on the 1st day after admission. Data presented as median ± IQR for all. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor.

For chemokines, although higher median levels of MCP-1, IL-8, and IP-10 were found in septic patients, these did not reach significance (*P*=0.51 and *P*=0.39). For IP-10, the difference between sepsis non-survivors and pre-operative control patients was almost significant (Kruskal-Wallis: *P*=0.17; Dunn's: *P*=0.06) (Figure 12).

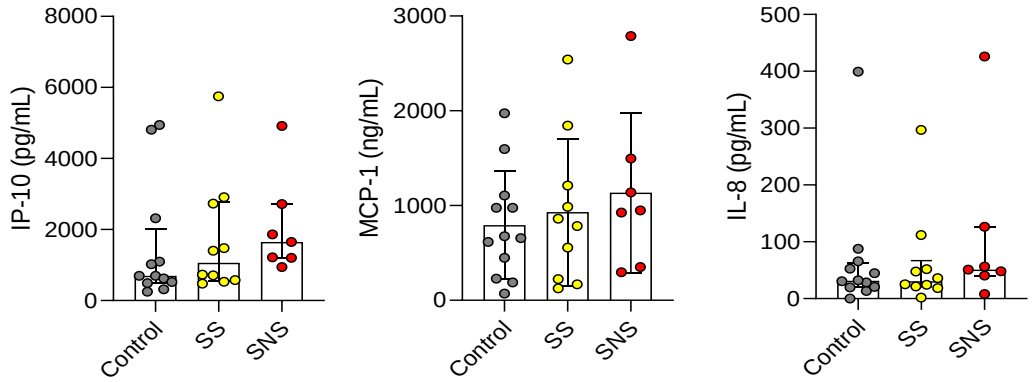

Figure 12. Serum levels of chemokines on Day 1. Chemokines were measured by multiplex assay in serum samples from patients with sepsis (divided into survivors (SS) and non-survivors (SNS)) and pre-operative control patients on the 1st day after admission. Data presented as median ± IQR for IP-10 and mean ± SD for MCP-1. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor.

2.3.4 Flow cytometry

Apoptosis and viability (using Annexin-V and 7AAD) of monocytes were measured by flow cytometry. HLA-DR expression, MMP (using TMRM), and mROS production (using MitoSox) were subsequently measured in live monocytes. In addition, Quantibrite HLA-DR was used to quantify HLA-DR fluorescence intensity by converting this signal into receptor expression per cell. Flow cytometry results for Day 1 were analysed and visualised. One sepsis non-survivor sample could not be analysed with flow cytometry, due to technical issues.

Live monocytes were identified as $CD14^+/7AAD^-$ while apoptotic monocytes were identified as $CD14^+/7AAD^-/Annexin\text{-}V^+$ cells. Gating cut-offs were determined based on FMO controls and heat-killed fully stained samples. Viability in all pre-operative control samples was good on Day 1 (>88.4%), but lower viability was found in some survivors (*n*=3) and non-survivors (*n*=1) (Supplementary Figure 1). There was no difference in viability among the three groups on Day 1 (*P*=0.89). Although the percentage of apoptotic monocytes was lower in septic patients on Day 1, especially in non-survivors, there was no difference between the three groups (*P*=0.30).

Due to variability in the percentage of live monocytes, and potential effects of cell death on our parameters, HLA-DR, MMP, and mROS signal were measured in live monocytes only. Monocyte HLA-DR expression, TMRM (as a measure of MMP) and MitoSox (as a measure of mROS) were visualised as the geometric mean of MFI signal (Figure 13).

No statistical difference between groups was seen in the TMRM signal (*P*=0.30). The MitoSox signal was significantly different on Day 1 between the three groups (*P*=0.03). Levels in septic patients, and especially in non-survivors, were higher compared to controls. After subsequent uncorrected Fisher's LSD multiple-comparisons analysis, a significantly higher MitoSox signal was found in sepsis non-survivors compared to pre-operative control patients (*P*=0.01).

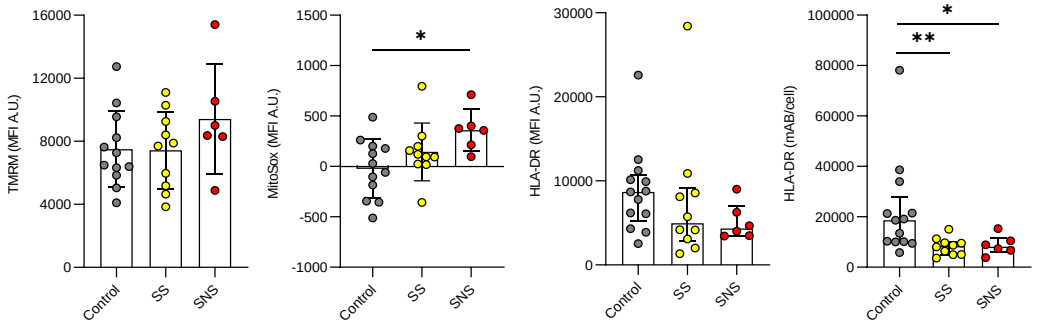

Figure 13. TMRM, MitoSox, and HLA-DR in live monocytes, and Quantibrite HLA-DR in total monocytes on Day 1. TMRM (measuring MMP), MitoSox (measuring mROS), HLA-DR, and Quantibrite HLA-DR (measuring quantified HLA-DR receptor expression/cell) signal was measured by flow cytometry of lysed whole blood samples from patients with sepsis (divided into survivors (SS) and non-survivors (SNS)) and pre-operative control patients on the 1st day after admission. TMRM, MitoSox and HLA-DR signal was measured in live monocytes $CD14^{+}/7AAD^{-}$) only. Data for TMRM and MitoSox presented as mean ± SD. Data for HLA-DR and Quantibrite presented as median ± IQR. MFI: Mean Fluorescence Intensity; A.U.: Arbitrary Units; SS: Sepsis Survivor; SNS: Sepsis Non-Survivor. *P<0.05; **P<0.01.

Monocyte HLA-DR expression in live cells trended higher in pre-operative control patients compared to septic patients, but differences did not reach significance (P=0.19). In addition to measuring monocyte HLA-DR expression in live cells, Quantibrite was also used to quantify HLA-DR signal into mAB/cell in a separate sample. The Quantibrite monocyte HLA-DR was significantly different between groups, with lower HLA-DR expression in sepsis survivors and non-survivors compared to control patients (Kruskal Wallis: P=0.007; Dunn's: P=0.03 and P=0.003, respectively), but without any differences between the two septic groups (P=0.76).

In this standardised method, borderline immunosuppression is established as 5,000-8,000 mAB/cell [424]. In the control group, one patient had a mAB/cell count of 5000-8000. For sepsis survivors, two patients had a count of 5000-8000 mAB/cell and two <5000 mAB/cell. For sepsis non-survivors, there were two patients with 5000-8000 mAB/cell and one with <5000 mAB/cell.

2.3.5 Correlation plots

Heat maps were used to plot the correlation between patient characteristics (age and severity scores), mitochondrial function (MMP and mROS production), monocyte function (HLA-DR expression), serum hormone levels, and a selection of inflammatory markers normally secreted by monocytes. This was done for these parameters on Day 1 for all three groups separately (Figure 14-Figure **16**).

2.3.5.1 Patient characteristics

The correlation between patient characteristics, including age and severity scores, with measures of mitochondrial and immune function, serum hormone levels, and inflammatory markers. Age and severity scores were positively correlated with each other in some of the groups. In pre-operative control patients, age and CCI were positively correlated (*P*=0.003). The same trend was observed for age and CCI and APACHE II scores in sepsis survivors, although this correlation did not quite reach significance (*P*=0.09 and *P*=0.07, respectively).

Age in sepsis non-survivors was negatively correlated with measures of mitochondrial and immune function (*P*=0.03 for TMRM and *P*=0.01 for HLA-DR expression). Similar observations were found for control patients, where age and measures of immune function and inflammation seemed negatively linked, although not always reaching significance (*P*=0.007 for HLA-DR; *P*=0.06 for Quantibrite HLA-DR; *P*=0.07 for TNF-α; and *P*=0.06 for IL-10). In addition, significant correlations were found for age and serum hormone levels, with a positive correlation between age and cortisol in control patients (*P*=0.002). In sepsis survivors, age was almost negatively correlated with insulin levels (*P*=0.09), and in non-survivors for fT_3 (*P*=0.09) and rT_3 (*P*=0.08).

Severity scores were linked with a variety of mitochondrial, immune and inflammatory markers. A weak negative link was found between SOFA scores and MitoSox in

survivors (*P*=0.09), and weak positive links for SOFA scores and HLA-DR expression, IP-10, and IL-8 in these same patients (*P*=0.06 for all). A similar positive correlation was found between SOFA scores and levels of IL-1β, IL-6, and IL-8 in non-survivors (*P*=0.04, *P*=0.01, and *P*=0.04, respectively).

Severity scores and serum hormone levels were linked for some of the groups. In survivors, a positive correlation between levels of fT_3 and rT_3, and severity scores CCI (*P*=0.04 and *P*=0.01), APACHE II (*P*=0.02 and *P*=0.02), and SOFA (*P*=0.01 and *P*=0.03) were found. In these patients, a negative correlation was found between CCI and APACHE II with insulin (*P*=0.009 and *P*=0.04), whereas CCI was positively correlated with adrenaline (*P*=0.05). SOFA scores in these patients were positively correlated with glucagon (*P*=0.005). Control patients had a positive link between CCI and levels of cortisol (*P*=0.02).

2.3.5.2 Mitochondria and the immune response

The correlation between mitochondrial function (MMP and mROS), HLA-DR expression (both by regular HLA-DR and Quantibrite HLA-DR) and serum inflammatory markers was assessed in control and septic patients (survivors and non-survivors).

Positive correlations were found between TMRM and MitoSox in control patients, and almost in sepsis survivors (*P*=0.02 and *P*=0.08, respectively). Whereas TMRM and HLA-DR had a weak negative link in sepsis survivors (*P*=0.06), there was an opposite positive correlation between the two in non-survivors (*P*=0.05). Similar trends were found for MitoSox and HLA-DR expression/Quantibrite HLA-DR expression. In control patients, a negative correlation was found between MitoSox and HLA-DR, Quantibrite HLA-DR, and IL-10 (*P*=0.08, *P*=0.02 and *P*=0.10, respectively). By contrast, in non-survivors, there was a positive link between MitoSox and HLA-DR (*P*=0.04), and MitoSox and IP-10 (*P*=0.06)

2.3.5.3 Hormones, mitochondria, and the immune response

Correlations between various levels of circulating hormones (noradrenaline, adrenaline, cortisol, fT_3, rT_3, and insulin) and mitochondrial, immune, and serum inflammatory markers were examined. These correlations were assessed separately for control patients, and sepsis survivors and non-survivors (Figure 14-Figure **16**).

Noradrenaline in pre-operative control patients was positively linked with HLA-DR, and almost with Quantibrite HLA-DR and TNF-α (*P*=0.05, *P*=0.08 and *P*=0.07, respectively). By contrast, there was a weak negative link between noradrenaline and levels of IL-8 in these patients (*P*=0.09). In survivors, noradrenaline was positively correlated with IL-10 (*P*=0.02), and almost with IFN-γ and and lL-1β (*P*=0.09 and *P*=0.05, respectively). The same trends were found for adrenaline and IL-10 and IFN-γ in these patients (*P*=0.08 for both). Positive correlations were found between adrenaline and TMRM, MitoSox, HLA-DR expression, IP-10 and almost for MCP-1 (*P*=0.04, *P*=0.02, *P*=0.03, *P*=0.04, and *P*=0.06, respectively) in non-survivors.

A weak negative link was found between cortisol and TNF-α and IL-10 in pre-operative control patients (*P*=0.10 and *P*=0.06, respectively). By contrast, a weak positive correlation between cortisol and IL-6 in sepsis survivors (*P*=0.08) was found. Similarly, in non-survivors, there was a positive correlation between cortisol and levels of TNF-α (*P*=0.007).

In non-survivors, a weak positive correlation was found between fT_3 and TMRM and MitoSox (*P*=0.05 and *P*=0.09, respectively). A similar trend was found in these patients for rT_3 and TMRM and MitoSox (*P*=0.04 for both). By contrast, rT_3 levels in survivors were weakly negatively correlated with MitoSox (*P*=0.10). In addition, correlations between thyroid hormones and markers of immune function and inflammation were found. A weak positive link between both fT_3 and rT_3 with HLA-DR expression was found in non-survivors (*P*=0.06 for both). Both fT_3 and rT_3 were positively correlated with IL-6 levels (*P*=0.002 and *P*=0.003, respectively), and fT_3

almost with IL-1β in control patients (*P*=0.08). In non-survivors, fT_3 and rT_3 were also positively correlated with IL-10 (*P*=0.04 and *P*=0.05), IP-10 (*P*=0.06 and *P*=0.02), and MCP-1 (*P*=0.09 and *P*=0.04).

Insulin was only positively correlated with MitoSox in sepsis survivors (*P*=0.05).

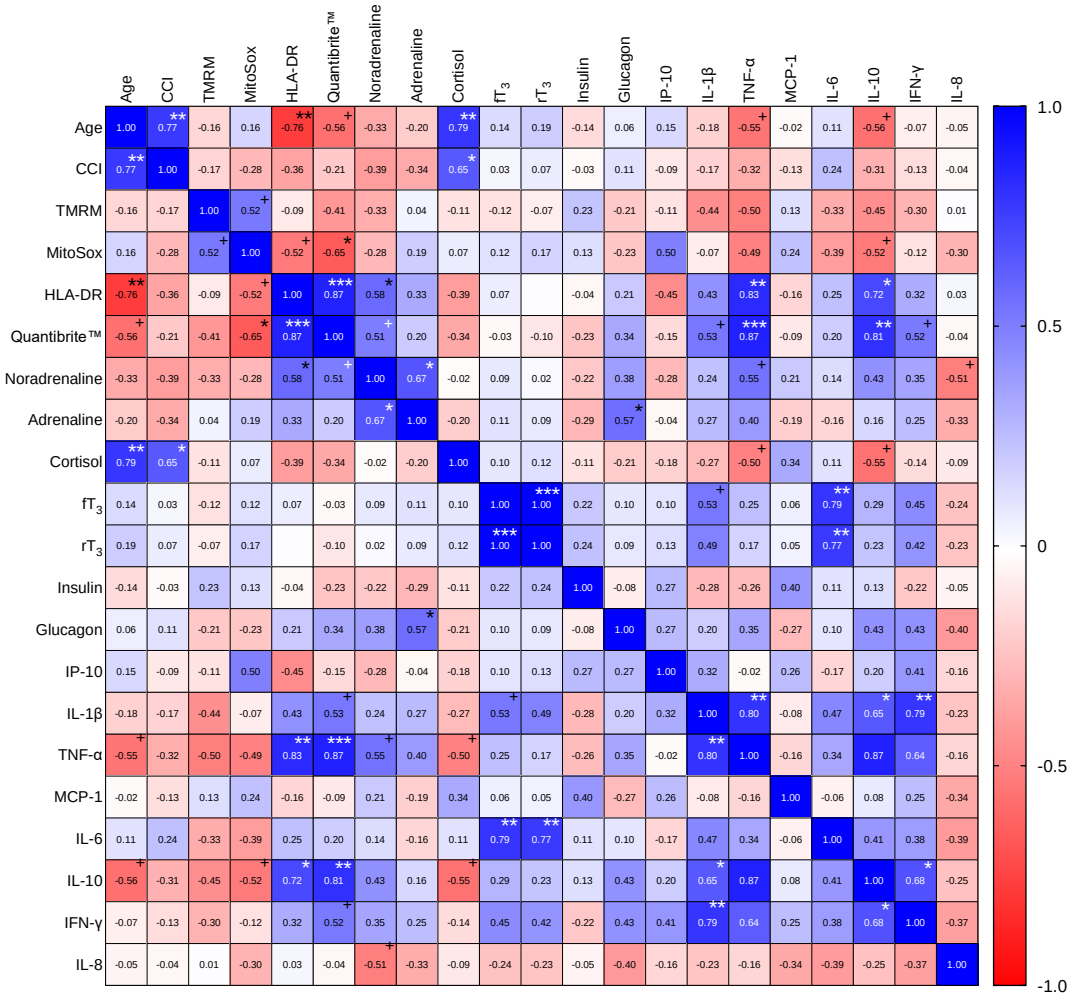

Figure 14. Correlation matrix for pre-operative control patients on Day 1. Pearson correlation coefficients between various patient characteristics, flow cytometry data on MMP, mROS and HLA-DR expression, hormone measurements and cytokines were calculated using Prism. This matrix only represents data from pre-operative control patients on the 1st day after admission. The intensity of the squares indicates the strength of the correlation, with negative correlations in red and positive correlations in blue. $^{+}P<0.10$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

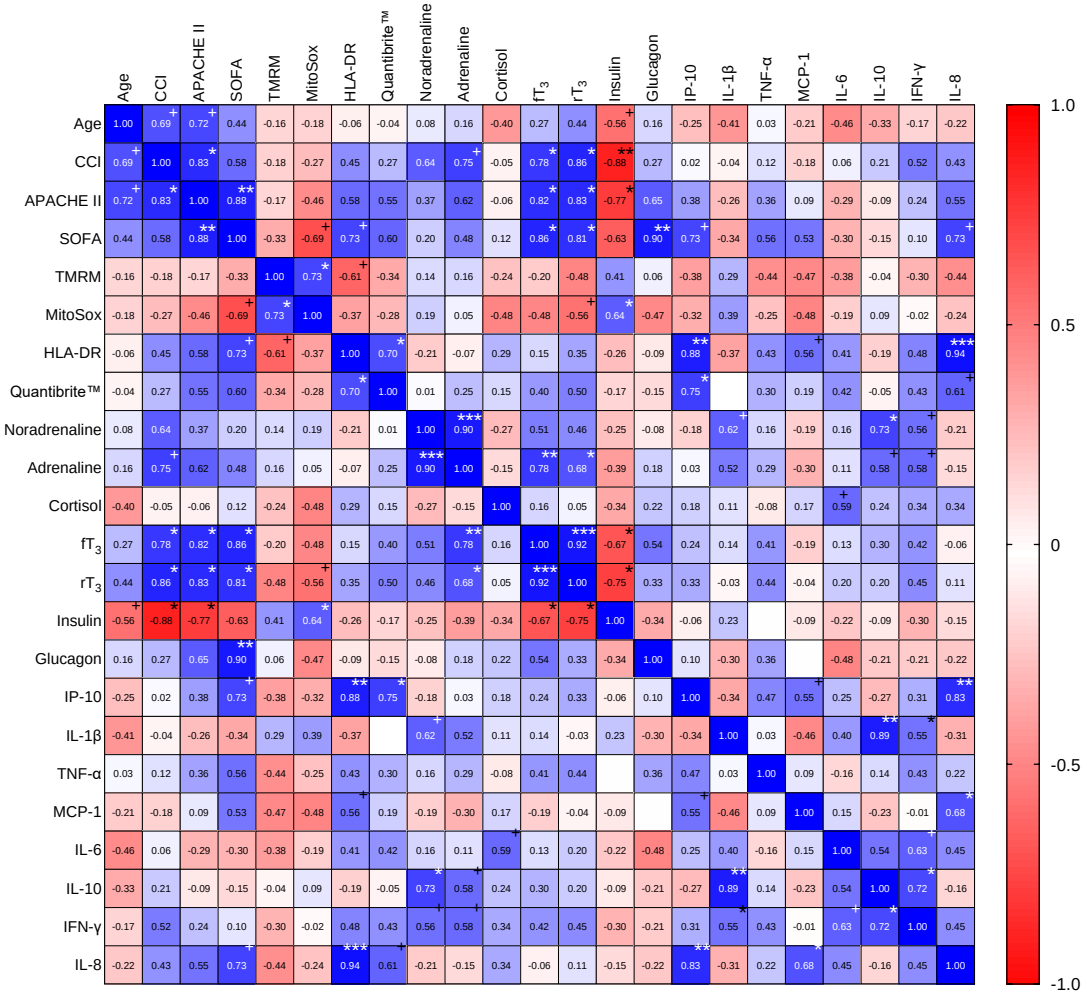

Figure 15. Correlation matrix for sepsis survivors on Day 1. Pearson correlation coefficients between various patient characteristics, flow cytometry data on MMP, mROS and HLA-DR expression, hormone measurements and cytokines were calculated using Prism. This matrix only represents data on the 1st day after admission from patients with sepsis that survived. The intensity of the squares indicates the strength of the correlation, with negative correlations in red and positive correlations in blue. $^{+}P<0.10$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

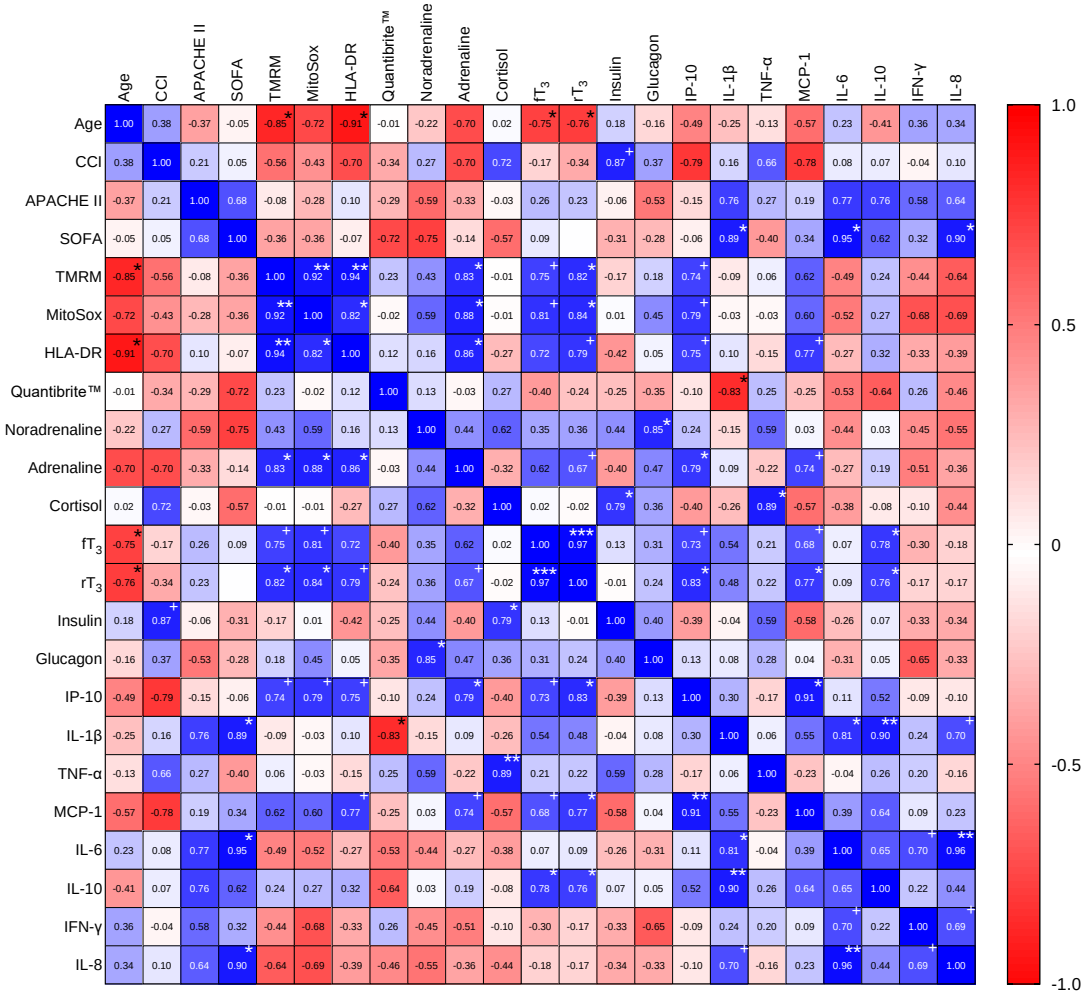

Figure 16. Correlation matrix for sepsis non-survivors on Day 1. Pearson correlation coefficients between various patient characteristics, flow cytometry data on MMP, mROS and HLA-DR expression, hormone measurements and cytokines were calculated using Prism. This matrix only represents data on the 1st day after admission from patients with sepsis that did not survive. The intensity of the squares indicates the strength of the correlation, with negative correlations in red and positive correlations in blue. $^{+}P<0.10$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

2.4 Discussion

The current study had a high mortality rate amongst patients with sepsis, and there were fewer men in the group of non-survivors. Pre-operative control patients were generally younger than the septic patients, especially compared to the non-survivors. Age was positively associated with severity scores, but negatively with measures of mitochondrial and immune function. In addition, there was a positive correlation with

cortisol levels in the control patients, but negative correlations with insulin in survivors, and both fT_3 and rT_3 in non-survivors. Although significant differences were only found for serum cortisol, some of the other measures of hormones, and mitochondrial and immune function were associated with severity scores.

Because of the relatively low numbers of patients recruited, there is likely to be a Type II statistical error, however the overall biological trend is similar. Transportation of samples and potential thawing and refreezing could have diminished detectable cytokine and chemokine levels, although these effects were limited by shipping samples on dry ice and aliquoting serum in small batches of 500 uL. Although samples collected at the ATTIKON University Hospital were processed immediately, samples from other hospitals had to be transported. As this happened at room temperature, delayed processing could have diminished measurable serum analytes. Due to lab closures during the SARS CoV-2 pandemic from March until July 2020, and very limited access for the months to follow, some of the analytes were measured after a prolonged storage time, possibly affecting sample quality. As normal curves were obtained and controls worked fine, we suspect a problem with the samples rather than a methodological issue with the consumables or equipment used.

mROS signal was higher in sepsis non-survivors compared to controls, and quantified HLA-DR expression was diminished in both septic groups compared to pre-operative control patients. MMP and mROS were positively linked in control patients. Interestingly, MMP was negatively associated with HLA-DR in survivors, but positively in non-survivors. Opposite directions of associations were also found for mROS and measures of immune function. While mROS was negatively associated with HLA-DR, quantified HLA-DR, and IL-10 in control patients, it was positively correlated with HLA-DR and IP-10 in non-survivors.

Catecholamines were positively linked to various measures of mitochondrial and immune function in the three groups, apart from the negative correlation between

noradrenaline and IL-8 in control patients. By contrast, cortisol was negatively linked with serum cytokines in control patients and sepsis survivors, but positively linked in non-survivors. Both fT_3 and rT_3 were positively linked with measures of mitochondrial and immune function, apart from the negative correlation between rT_3 and mROS in survivors. Insulin had a positive correlation with mROS, but no associations were found for glucagon.

2.4.1 Baseline characteristics and measurements

2.4.1.1 Patient characteristics

The age of septic patients enrolled in the current study was high, with an average age of 78 years for survivors and 87 years for non-survivors. They were significantly older than the pre-operative controls. Compared to the recent EPIC II study on prevalence and outcomes of infection in ICUs worldwide with a reported mean age of 61 years [425], the patients in my cohort were very elderly. The immune system deteriorates with age, even in health [426]. The response to infection and other stressors will also be affected as evidenced in a mouse study by Turnbull and colleagues [427].

Most septic patients are male; Vincent et al. (2020) reported 60.4% of patients to be male in their point prevalence study of ICU patients [425]. Although the sepsis survivors in the current study were predominantly male, the non-survivor group were predominantly female. This may be a chance finding, especially as numbers recruited were low, but could represent a potential confounder as sex differences are known to impact upon the body's response to stress and infection [428].

In my study, the mortality rate was 41.2%, with all patients dying within the first 11 days after ICU admission. This is higher than the in-hospital mortality rate of 30% reported in the EPIC II study [425], though, as mentioned, the average age of the septic patients in the current study was considerably higher. As per EPIC II, most

patients were admitted with respiratory chest infections, followed by urinary tract infections, abdominal infections, and primary bacteraemia.

2.4.1.2 HLA-DR expression

The reduced monocyte HLA-DR expression in both survivors and non-survivors did not reach significance. However, the quantified HLA-DR signal was significantly lower in septic patients compared to controls (P=0.007), with no difference between survivors and non-survivors (P=0.76) (Figure 13). Decreased expression of monocyte HLA-DR is a well-known marker of immune failure during sepsis, and is associated with an increased risk of secondary infections and mortality. An overview of studies evaluating the prognostic value of monocyte HLA-DR expression by Venet and colleagues revealed that initial monocyte HLA-DR is often not prognostic, whereas monocyte HLA-DR measured on ICU Days 3-4 is associated with poor outcomes, including an increased risk of secondary infection [429].

The differences in results between the two monocyte HLA-DR markers in the current study could be explained by the differences in dyes. Quantibrite HLA-DR incorporates anti-monocyte PerCP-Cy5.5 that recognises Fc Receptor (FcR)-1 (CD64), in addition to monocyte anti-CD14, studying a slightly different monocyte population. In addition, Quantibrite HLA-DR reacts with the HLA-DR epitope and does not cross-react with other MHC-II molecules, such as HLA-DQ or HLA-DP. Also, Quantibrite HLA-DR does not incorporate a live/dead marker, while 7AAD was added as a marker of cell death to the other HLA-DR sample.

2.4.1.3 Serum chemo- and cytokines

Patient serum samples were frozen and then later transported on dry ice from Athens to UCL for multiplex analysis of various cytokines and chemokines. Although most chemokines, and pro- and anti-inflammatory cytokines, seemed higher in sepsis

patients, and especially in non-survivors, no significant differences were found. By contrast, levels of pro-inflammatory IL-2 and anti-inflammatory TGF-β1 trended lower in sepsis survivors compared to control patients and non-survivors.

Changes in cytokine levels in sepsis have been well documented. Serum levels of proinflammatory cytokines such as TNF-α, IL-1β, IL-2, IL-12, and IL-6 are generally – but not always - increased in septic patients [430-432], with levels often correlated with disease severity, septic shock, organ failure, and increased risk of death [433-436]. Anti-inflammatory cytokines such as IL-4, IL-10, TGF-β, and IL-1ra, also rise simultaneously.

The magnitude of change in cytokine levels, if any, does however depend on timing of sampling in relation to the onset of illness, the severity of illness, and the underlying infectious cause. For example, a recent study on circulating IL-6 in patients with severe or critical disease found significantly higher levels in patients with cytokine release syndrome and sepsis, and patients with Acute Respiratory Distress Syndrome (ARDS) when compared to COVID-19. Despite these elevations in sepsis and ARDS, cytokine blockade has not been effective in these patients, leaving unanswered questions about the mechanistic role of these increased levels [437].

In addition, mixed effects have been published on the production and release of IL-1β in sepsis. An increase in serum levels, especially in non-survivors, has been reported [431, 438], but also defective IL-1β production in septic shock [438, 439]. A rise is seen in both serum and Th1 cell mRNA levels of IFN-γ in septic patients compared to non-septic controls, however, patients in septic shock had lower levels compared to non-shock septic patients [440]. Levels of IL-10 were higher in septic shock compared to non-shock patients [441], with upregulation being associated with worse prognosis [430]. Higher levels of TGF-β occur early during sepsis in sepsis survivors, with lower levels at 10 days, while the opposite was seen for non-survivors [442]. Serum levels of the chemokines IL-8 and MCP-1 were higher in sepsis patients,

especially in non-survivors [430]. These higher levels of IL-8 also correlated with an increased risk of mortality, whereas MCP-1 may play a role in the development of organ dysfunction in sepsis [443, 444]. Levels of IP-10 are considered as a biomarker to predict disease severity in patients with acute respiratory infection [445].

It is also important to state that these serum analytes are produced and released by a variety of cells and are not specific to monocytes, but rather give a general overview of the inflammatory response within the circulation.

2.4.1.4 Apoptosis and cell death

Viability in most samples was good, without any differences between groups. There were no significant differences between groups in the degree of apoptosis, although the percentage of apoptotic monocytes trended lower in non-surviving septic patients. Some samples had lower percentages of viable cells and higher percentages of apoptotic cells, potentially due to transportation of samples from other hospitals and delays in processing. While delayed neutrophil apoptosis and increased apoptosis of NK cells, $CD4^+$ and $CD8^+$ T-cells, B-cells, and DCs are common markers of sepsis-induced immunosuppression [17, 42], effects on monocyte are less well studied.

Giamarellos-Bourboulis et al. (2006) found an early increase in monocyte apoptosis associated with improved survival in septic patients [446], but underlying mechanisms were not studied. Although consistent with our finding that apoptosis might be lower in those who go on to die, others have questioned whether monocyte apoptosis could indicate other factors causally associated with mortality [447]. In addition, this study reported an implausibly high percentage of apoptotic monocytes (37.7 ± 5.8% for survivors and 11.7 ± 8.2% for non-survivors on Day 1).

By contrast, an earlier study by Adrie et al. (2001) reported monocyte apoptosis of 4.5 ± 2% and 7.4 ± 2.9% in sepsis survivors and non-survivors, respectively, and 3.1 ±

1.5% in control patients [135]. They found a significant increase in the percentage of dead cells (apoptotic plus necrotic) between control subjects and septic patients within the first 3 days of admission, but no differences at 7-10 days and at ICU discharge, and without differences between survivors and non-survivors. This highlights that temporal changes in apoptosis and timing of sample collection are crucial. The duration of illness before enrolment in these studies may introduce enough variability to make comparisons difficult.

2.4.1.5 Mitochondrial function

TMRM, used as a measure of MMP, trended higher in septic non-survivors compared to both control patients and septic survivors, but differences did not reach significance. The MitoSox signal, indicating mROS production, was higher in sepsis, with a significant difference between sepsis non-survivors and control patients. There is extensive evidence of sepsis-induced effects on mitochondria, including altered MMP and increased mROS production. These have been found in a wide range of tissues and cell types from clinical studies and animal models, including PBMCs (monocytes and lymphocytes) and monocytes specifically [129].

The MMP usually reflects electron flow through the complexes of the ETC, ultimately facilitating ATP generation [448]. In addition to ATP generation, mitochondria are involved in many other functions, including mROS generation. In most cells, ROS are primarily secreted via this route. However, in phagocytic immune cells, ROS can also be generated in large quantities by NADPH oxidase. Although regulated mROS plays an important signalling role and affects immune cell function and microbial clearance, uncontrolled generation can have deleterious effects on cellular functions [111].

The increase in mROS in the current study is in line with increased mROS generation and oxidative stress reported during sepsis that likely contributes to organ dysfunction [113, 134]. TMRM and MitoSox were positively correlated. Due to the deleterious

effects of excessive mROS, it could be argued that a lower MMP seen in septic survivors compared to non-survivors may, at least in part, prevent excessive mROS formation. This may be related to increased mitochondrial uncoupling [119]. However, two studies have reported an increase in the percentage of impaired and depolarised monocytes during sepsis [134], with higher levels in non-survivors [135]. These differences had disappeared in survivors by ICU discharge, highlighting the temporal changes seen in MMP during sepsis. In a paediatric sepsis study, Weiss and colleagues (2015) found no change in MMP of PBMCs, but a raised MMP at days 1-2 was associated with the duration of organ injury [147].

Weiss and colleagues (2021) also performed another study with PBMCs and concluded that mitochondrial measurements, including respiration and Citrate Synthase (CS) activity, varied with changes in immune cell composition in children with and without sepsis [151]; they considered that differences in PBMC mitochondrial measurements between sepsis patients and controls were at least partially attributable to the effects of sepsis. One should be careful comparing outcomes in PBMCs in general with specific effects in monocyte subsets.

2.4.2 Mitochondria and the immune response

A significant positive correlation between TMRM (as indicator of MMP) and MitoSox (as indicator of mROS) was found in all patients. A higher MMP is known to increase production of mROS [449] which, in turn, is a potent initiator of the innate immune system with activation of the NLRP3 inflammasome [450], degradation of pathogens during phagocytosis, and modulation of TLR pathways and downstream signalling [451]. However, excess mROS could also have potentially unwanted consequences and damage organelles or cells.

Strikingly, correlations between mitochondrial function and HLA-DR expression pointed in opposite directions for septic non-survivors compared to survivors and

control patients. TMRM was negatively correlated with HLA-DR expression in sepsis survivors, but positively correlated in sepsis non-survivors. Similarly, for MitoSox, there was a negative correlation with HLA-DR and Quantibrite HLA-DR in control patients, but a positive correlation in sepsis non-survivors. Although it was hypothesised that HLA-DR expression by monocytes is affected by mitochondrial dysfunction, it was only possible to study associations rather than causation.

Belikova and colleagues (2007) found that basal rates of O_2 consumption in PBMCs from septic patients and controls was negatively correlated with HLA-DR expression, but the percentage increase in O_2 consumption after ADP stimulation was positively correlated [146]. However, they studied total PBMCs (lymphocytes and monocytes) rather than monocytes, and incubated healthy cells with plasma taken from septic patients at different timepoints, rather than measuring PBMCs from septic patients directly. The distinct direction of effects in the current study highlights the importance of stratifying patients into distinct groups for analysis and suggests a differential regulation in patient groups.

In sepsis non-survivors, both TMRM and MitoSox were positively correlated with circulating IP-10, which is in line with correlations with HLA-DR expression. In control patients, there was a negative correlation between MitoSox and IL-10 in serum. Anti-inflammatory IL-10 prevents accumulation of dysfunctional mitochondria and production of mROS via the induction of mitophagy in bone marrow-derived macrophages upon LPS stimulation [452]. This could explain the negative correlation found in control patients, with dysregulation especially in sepsis non-survivors.

mROS is an activator of the NLRP3 inflammasome, and subsequent production of IL-1β [450]. No significant correlation in the current study was found between MitoSox and serum IL-1β. However, levels found in serum are an accumulation of cytokines produced by a variety of cells and do therefore not necessarily represent IL-1β production by monocytes. No other significant correlations were found between

measures of mitochondrial function and serum inflammatory markers. Merz et al. (2017) also found no correlation between parameters of enzymatic activity or ATP content and cytokines (IL-1β, IL-6 and IL-10) in monocytes from septic patients [150].

Another possible explanation for these differences between control patients and survivors and non-survivors with correlations in opposite directions is the dependency of monocytes on glycolysis rather than OXPHOS during activation. While inhibition of glycolysis decreased production of pro- and anti-inflammatory mediators in LPS-stimulated monocytes, a reduction in OXPHOS inhibited pro-inflammatory cytokines in monocytes and macrophages [180, 453, 454].

2.4.3 Hormones, mitochondria, and the immune response

2.4.3.1 Catecholamines

In the current study, serum noradrenaline levels surprisingly did not show any significant difference in patients with sepsis compared to control subjects. Median noradrenaline levels in sepsis survivors fell within the normal range (0.14-0.86 ng/mL) of non-stressed healthy subjects, but much higher levels would have been predicted in these critically ill patients. Likewise, median serum adrenaline levels remained within the normal range in septic patients, and did not differ from controls. Adrenaline even fell below the detectable range in many patients, despite high quality standard curves. Shock states and critical illness should result in early and marked elevations in both noradrenaline and adrenaline [66], the magnitude of which is greater in non-survivors [342]. Although sedation of patients on the ICU could have contributed to suppressing the sympathetic nervous system and subsequent production of catecholamines, this is unlikely as most of the patients were not sedated. It is difficult to interpret these results and a methodological problem must be considered as discussed at the start of this paragraph.

Although no significant correlations were found between noradrenaline and TMRM or MitoSox, there was a positive correlation between adrenaline and both these measures of mitochondrial function in non-survivors. Catecholamines generally mobilise energy substrates for oxidation and conversion into ATP, rapidly increasing mitochondrial activity in various cells, including hepatocytes [190]. More specifically for monocytes, LPS exposure increased basal and maximal O_2 consumption and glycolysis in catecholamine-trained cells, while the opposite was observed in naïve monocytes [193]. This suggests that adrenaline's effects on mitochondrial function and metabolism depend on the activation state of the cell. This could, at least partially, explain the differences found between control patients and patients with sepsis.

Previous studies with cardiomyocytes and adrenaline showed a simultaneous increase in mitochondrial activity, ROS formation, and antioxidant capacity [455, 456]. Increased oxidative stress with decreased GSH was also seen after exposure of gut tissue to adrenaline [457]. A significant correlation between adrenaline, TMRM, and MitoSox was however only found in septic non-survivors in the current study. This suggests that adrenaline's lack of effects on MMP and mROS production in control patients and septic survivors might be explained by effects on other mechanisms such as uncoupling or antioxidant capacity.

There were mixed positive and negative correlations between plasma catecholamines and both pro- and anti-inflammatory mediators. No clear picture emerged to suggest a direct impact of either noradrenaline or adrenaline on monocyte cell function. However, it should be stressed that serum analytes are produced by a wide range of cells and not by monocytes specifically.

In another study of septic patients, a higher dose noradrenaline infusions were correlated with a more pro-inflammatory cytokine balance, whereas β-blocker use was associated with an overall anti-inflammatory balance [196]. They also used a human endotoxemia model to demonstrate that noradrenaline enhanced IL-10

production and attenuated the release of the pro-inflammatory IP-10. They concluded that the effects of noradrenaline on the immune system are mainly β-AR dependent, in particular β_2-AR, resulting in reduced expression of pro-inflammatory cytokines. Catecholamines have varying affinity for the α- and β-ARs, depending on their concentration [69]. This could, at least in part, explain the discrepancies between the current study and the literature.

HLA-DR and noradrenaline might be correlated via IL-10, which is a known depressant of monocyte HLA-DR. Downregulation of HLA-DR on classical monocytes was observed in cardiogenic shock patients and correlated with the noradrenaline dose used for treatment [458]. On the other hand, *in vitro* experiments in monocytes and whole blood found no effect of noradrenaline on HLA-DR expression [459, 460]. This suggests that noradrenaline might not affect HLA-DR directly, but rather have a common effector, or that levels of IL-10 influence HLA-DR expression.

2.4.3.2 Cortisol

Cortisol levels in both septic groups were higher compared to control patients, but only reached statistical significance in the non-survivor group. Levels of cortisol during sepsis are normally elevated, but diurnal variation is frequently lost and there is often poor responsiveness to exogenous ACTH stimulation [78-80]. Significant differences were not found between sepsis survivors and non-survivors, although the degree of increase in cortisol has previously been associated with the severity of illness [461].

No significant correlations were found between cortisol and measures of mitochondrial function in any of the groups. Generally, glucocorticoids stimulate mitochondrial metabolism by mobilising energy substrates [225]. In addition, glucocorticoids can affect mitochondria by direct regulation of gene expression, and by affecting kinase signalling pathways [200, 219, 462, 463]. Glucocorticoids affect mitochondrial function in a time- and dose-dependent manner. Differences in duration

between disease onset and admission could have outbalanced glucocorticoid-induced effects in sepsis.

In sepsis survivors and in non-survivors, positive correlations were found between cortisol and IL-6 and TNF-α, respectively. Glucocorticoid effects are generally anti-inflammatory [356]. Although cortisol levels were higher during sepsis, glucocorticoid resistance may have developed in these patients with suppressed GR expression and sensitivity, aggravating inflammation [82, 355].

As the current study looked at correlations rather than causative effects, changes in serum cytokines could affect the production and release of cortisol. Indeed, release of inflammatory cytokines activate the HPA axis and cortisol production [76, 464]. Excessive cytokines could also reduce the number and binding affinity of the GR [82].

2.4.3.3 Thyroid hormones

No differences were detected between any of the groups for both fT_3 and rT_3. Very high levels of both hormones were detected in one control patient who, to our knowledge, did not receive any thyroid hormones as part of their medication. Serum levels of fT_3 exceeded the normal range of 3.0-7.9 pg/mL in both control and septic patients. Similarly, levels of rT_3 exceeded the normal range of 91-221 pg/mL for all groups [465]. In septic patients, although not measured, this could potentially be explained by a reduced concentration of thyroid hormone binding proteins [90, 91]. Although levels of serum fT_3 and rT_3 did not differ, the ratio between the two seemed to decrease in sepsis, especially in non-survivors. This is in line with a decrease in circulating levels of fT_3, whereas levels of rT_3 increase, likely due to altered peripheral conversion of T_4. The degree of these changes usually reflects severity of illness [89].

Whereas rT_3 was almost negatively correlated with MitoSox in survivors, both fT_3 and rT_3 were positively correlated with TMRM and MitoSox in non-survivors. Thyroid

hormones, and especially T_3, are generally known for their hypermetabolic effects via genomic and non-genomic pathways [466, 467]. More specifically, T_3 has been associated with increased mitochondrial respiration but with a reduced efficiency potentially due to uncoupling [468, 469].

T_3 treatment led to increased mROS production in both *in vivo* and *in vitro* models [272, 470]. Oxidative stress was confirmed by increased expression of antioxidant defence genes. By contrast, rT_3 is less metabolically active, although its effects on MMP and mROS have not been studied. By competitive binding of rT_3 to its receptor, the induction of mitochondrial activity and metabolism by fT_3 could be prevented. The correlation between thyroid hormones and measures of mitochondrial function in control patients and sepsis survivors was absent, potentially due to changes in thyroid receptor expression or binding [471]. The fall in fT_3/rT_3 ratios in non-survivors could have resulted in diminished uncoupling of the respiratory chain and antioxidant defences, resulting in higher MMP and mROS production in these patients.

In non-survivors, both fT_3 and rT_3 were positively correlated with HLA-DR expression. Although thyroid hormones have genomic actions, its effect on HLA-DR expression are less well studied. One study in HELA cells showed that T_4 and T_3 enhanced IFN-γ induced HLA-DR levels, whereas reverse T_3 did not [472]. However, no significant correlations were found between thyroid hormones and serum INF-γ in these patients. It must be noted that serum INF-γ is not merely a reflection of production by monocytes, but rather an indication of the general inflammatory response.

In addition to effects on HLA-DR, positive correlations were found for both fT_3 and rT_3 with IL-6 levels, and for fT_3 with IL-1β in control patients. IL-1β inhibits deiodinase enzyme activity and affects thyroid hormone metabolism in HepG2 cells [473]. It also modulates TR expression in the same cell line [474]. While significant correlations for survivors were found, both fT_3 and rT_3 were positively correlated with IP-10, MCP-1,

and IL-10 in non-survivors. However, surprisingly, anti-inflammatory IL-10 was also correlated with these hormones.

In general, increased thyroid hormone levels result in an amplification of the pro-inflammatory response of innate immune cells [87, 475]. However, one study found that IL-6 and IL-10 had a significant negative correlation with fT_3 in critically ill patients [476]. Effects of rT_3 on immune function have been less well studied.

2.4.3.4 Insulin

Insulin levels in sepsis survivors trended lower compared to control patients and sepsis non-survivors, but differences did not reach statistical significance. Circulating insulin levels may initially fall during sepsis, and this can be followed by the development of insulin resistance, which manifests itself as hyperglycaemia and hyperinsulinaemia [59]. Indeed, high glucose levels were found in the septic patients, especially in non-survivors. Serum insulin in non-survivors was highly variable, with levels in three patients outside of the normal range of 2-20 microU/L. These outliers did not receive insulin as part of their medication or treatment.

Insulin was positively correlated with MitoSox in sepsis survivors. Hyperglycaemia induces superoxide production by the mitochondrial respiratory chain. The effects of insulin on glucose control, rather than direct effects, may protect against mitochondrial abnormalities [79]. This is however contrary to the positive correlation observed in my study. In line with my results, a study found increased ROS production on acute exposure to insulin in LPS-stimulated primary human monocytes, although the ROS were not mitochodria-specific [403].

No correlations were found in the current study between insulin and MMP. Insulin can modify mitochondrial function, with increased expression of mitochondrial proteins, enhanced MMP, higher oxidative enzyme activity and elevated ATP synthesis in both

muscle and sensory neurons [305, 309, 477]. These effects were diminished in subjects with insulin resistance [478]. While insulin resistance during sepsis could explain the lack of correlation in septic patients, it does not explain the lack of correlation in control patients [306].

No other correlations between insulin and measures of immune and inflammatory function were found. A recent review on the immunomodulatory effects of insulin argues that it exerts both pro- and anti-inflammatory effects, depending on the activation state of cells [378]. Although anti-inflammatory effects of insulin have mostly been reported, recent findings implicate a more involved role for insulin in shaping the immune response during an infection. These divergent roles could be explained by (i) the transcriptional activity of the insulin receptor, (ii) ratios between specific isoforms of proteins in the insulin signalling pathway, (iii) insulin signalling crosstalk with other immunological pathways, and (iv) dose and time-dependent effects.

2.4.3.5 Glucagon

Although glucagon levels trended lower in sepsis survivors compared to control patients and sepsis non-survivors, differences did not reach significance. Serum levels of glucagon in control patients fell within the normal range (40-120 pg/mL), whereas levels in sepsis survivors fell below the normal range. In some sepsis survivors, however, glucagon was not detectable, and this may potentially skew the results. Glucagon normally rises in acute illness to oppose the role of insulin in elevating sugar levels and other energy substrates [71]. This elevation in glucagon has previously been associated with disease severity and mortality sepsis [96, 97].

In the current study, no associations were seen between glucagon and measures of mitochondrial function. Increased glucagon to insulin ratios may be responsible for a decrease in glycolysis, and increase in OXPHOS in rat hepatocytes [286]. More specifically, glucagon is reported to stimulate mitochondrial activity, increasing MMP

and ATP formation in liver cells [282, 284, 285, 479, 480]. However, the literature on its effects on immune cells is limited.

Potentially, a glucagon-induced increase in mitochondrial activity and ATP formation would increase mROS production. Glucagon could also potentially increase mROS production by induction of hyperglycaemia [481, 482]. Despite this evidence, glucagon reduced mROS in a stimulated rat neuronal cell line model [483]. Glucagon also inhibited superoxide production by polymorphonuclear cells [367].

No correlations were found between glucagon and measures of immune function and inflammation for any of the groups in my study. Limited studies have investigated glucagon's effects on immune cell function. The general view is that it has anti-inflammatory effects [484], with decreased production of cytokines by Th2 cells [485, 486], decreased NK-cell activity [370, 371], and reduced neutrophil migration, chemotaxis, and adherence to endothelial cells [368, 487]. These effects increased susceptibility to sepsis in diabetic mice [368].

2.5 Strengths and limitations

This observational study in septic patients intended to map temporal endocrine changes, inflammatory markers, mitochondrial and immune function of monocytes, and the correlation between these parameters in sepsis.

The number of patients to be included in the current study was decided based on a previous study on endocrine and inflammatory changes in sepsis survivors and non-survivors. However, that predetermined number could not be achieved within the time given for this secondment in Athens, even after extension. The small number of patients makes it impossible to analyse subgroups of patients with this heterogenous condition. There is a chance of encountering type II errors due to these low patient

numbers. The pre-operative patients used as reference were not precisely sex- and age-matched to the septic patients.

It was also planned to examine the temporal endocrine, mitochondrial, immune, and inflammatory response. However, due to high mortality rates and discharge of patients, this became impossible after Day 1. The days included in the current study design, with Day 7 being the longest after hospital admission, are still relatively early, meaning prolonged critical illness was not examined. Including days beyond those already used in the current study would have been impossible, due to even higher numbers of patients that died or were discharged.

Sepsis requires medical treatment that cannot be ethically withheld from patients. In addition to sepsis-related treatment and medication, septic patients often have many comorbidities, all with their own set of prescribed treatments. Such medications include glucocorticoids, catecholamines, insulin, and β-blockers, and these could have confounded parameters that were studied in these experiments. Patients receiving these sepsis-related interventions are often more severe cases and can therefore not be excluded from the study, as this would lead to an inaccurate representation of the total population of sepsis patients.

Another factor making studying endocrine effects difficult, in addition to administration of exogenous hormones in patients, is the fact that hormones are not released uniformly throughout the day. Instead, they respond to various stressors and to the metabolic state of patients. However, to avoid effects of fluctuations throughout the day, blood was drawn every day between 8.00-9.00 am to keep these effects as constant as possible. Furthermore, as mentioned above, diurnal rhythms of hormone secretion are often lost in critical illness.

It was attempted to study as many endocrine, immune, and mitochondrial changes within the same set of patients as possible. Due to the available equipment in the host

lab in Athens, this had to be limited to some of the parameters included. The flow cytometer allowed detection of only five fluorochromes simultaneously, but due to the wide excitation of MitoSox in particular, this had to be limited to four. This made it impossible to study monocyte subsets or other cell types. In addition, measurements of mitochondrial and immune cell function also had to be limited.

By not including measurement of stimulating hormones and precursors, it cannot be concluded which steps within endocrine pathways were affected. Inclusion of more mitochondrial parameters, such as complex activity assays, antioxidant activity, and respirometry for measurement of O_2 consumption, uncoupling and ATP production, would have given a more complete overview of all endocrine-induced effects. However, due to the already extensive nature of current measurements, the most relevant measures had to be selected.

For MitoSox specifically, the question remains as to whether this dye is mitochondrial co-specific for phagocytic cells. MitoSox normally specifically targets mitochondria where it is rapidly oxidised by superoxide. The manufacturer's validation experiments for mitochondrial specificity have not included phagocytic cells, where the majority of ROS is produced by NADPH oxidase. In these cells, MitoSox could be exposed to non-mitochondrial ROS outside of the mitochondria, be oxidised, and emit a non-mitochondrial specific fluorescent signal.

2.6 Summary and conclusions

This *in vivo* study examined the link between various circulating hormone levels, mitochondrial and immune cell function of monocytes, and serum inflammatory markers from septic patients and pre-operative controls on Day 1 after admission.

Interestingly, opposite directions of correlations were found for some measurements. For example, MMP was positively associated with HLA-DR expression in survivors,

but negatively in non-survivors, without significant differences in MMP or HLA-DR between these two groups. Similarly, mROS was negatively associated with HLA-DR, quantified HLA-DR, and IL-10 in control patients, but positively with HLA-DR and IP-10 in non-survivors, with higher mROS levels in sepsis patients, especially in non-survivors. This highlights potential dysregulation of mitochondrial function and HLA-DR expression during sepsis, especially in non-survivors. This suggests that affecting mitochondrial function might be beneficial in some patients, but could be detrimental in others, depending on the dominating inflammatory or immunosuppressive phase [488].

Catecholamines are currently being used in septic shock for haemodynamic stabilisation. Whereas noradrenaline is recommended as the drug of first choice, adrenaline is recommended for those with an inadequate response to noradrenaline and vasopressin, or for those patients with septic shock and suppressed cardiac function [8]. Whereas noradrenaline has previously been suggested as a driver of sepsis-induced immunosuppression, a correlation was found predominantly with pro-inflammatory mediators. Again, potential effects on patient outcome will depend on disease state and immune function, but my results do not warrant a search for alternative vasopressors or administration of β-blockers as suggested by others [196].

While cortisol was negatively correlated with TNF-α and IL-10 in control patients, it was positively correlated with IL-6 and TNF-α in sepsis survivors and non-survivors. Cortisol levels were significantly higher in non-survivors compared to control patients; this could indicate differential regulation depending on dose. Indeed, variable dosing effects have recently been studied in COVID-19, and have also been argued to be a potential explanatory factor in controversial effects in sepsis [489, 490].

Other explanatory factors, although not studied, include differences in activation state of cells, or receptor expression and sensitivity. Due to these controversial effects, glucocorticoids are currently only suggested to lower vasopressor requirement in

septic shock [8]. Differences found in the current study highlight the importance of stratifying patients based on glucocorticoid levels and sensitivity, and disease state and immune function.

Whereas generally positive associations have been found between both fT_3 and rT_3 with mitochondrial measurements, HLA-DR, and inflammatory markers in control patients and non-survivors, rT_3 was almost negatively associated with mROS in sepsis survivors, without any differences in rT_3 serum levels. These differential directions suggest altered receptor expression or sensitivity of cells. Administration of fT_3 or rT_3 may be beneficial in those who are immunosuppressed but could potentially induce excessive mROS formation in others. Thyroid hormones are not currently used routinely in clinical practice but have been studied in critical illness. Studies with substitution doses of T_4 and T_3 have been unable to increase circulating plasma levels and reveal any benefits. However, on the other hand, supraphysiological doses could lead to overtreatment and further TSH suppression. An intervention study with TRH re-established normal thyroid levels but did not elicit any changes in metabolic response [416].

The positive link between insulin and mROS in survivors, without any correlations with other measures of mitochondrial or immune functions, makes it difficult to draw conclusions. The same applies to glucagon. The use of insulin, especially its effect on normalising blood glucose levels, has been shown to benefit immune function [491]. However, its effects on morbidity and mortality has been controversial and its use in clinical practice is still a matter of debate [89].

The current study was most likely underpowered, making it difficult to draw conclusions and adjust for interactions with other endocrine and immunomodulating drugs and treatments, and potential confounders. Studying endocrine effects on mitochondrial and immune function during sepsis is highly complex due to the heterogenous and biphasic nature of the disease, and interplay between various

hormones. For example, catecholamines and glucocorticoids increase insulin resistance [89]. Altogether, the current study highlights the importance of stratifying patients based on endocrine levels, sensitivity of cells, and immune status.

In addition, associations between measurements were looked at, rather than studying causative effects of hormones. Conclusions on the direction of possible associations can therefore not be drawn. To reliably study the association between hormone changes and mitochondrial disruption in these critically ill patients, one would have to include more patients, especially when subsequently dividing patients with this heterogenous condition into subgroups. Another option would be to isolate PBMCs or monocytes from these patients, incubate them in their own serum, manipulate the levels and effects of these hormones by adding them to the cells, or blocking their receptors. In future experiments it would be interesting to include more measures of mitochondrial function, including respirometry.

Chapter 3 Catecholamine- and glucocorticoid-induced effects on monocyte mitochondrial and effector function; an *in vitro* model of infection

3.1 Introduction

In vitro stimulation studies were executed with Heat-Killed Bacteria (HKB) and a selection of stress hormones, namely adrenaline, noradrenaline, and hydrocortisone. These were selected based on (i) changes in endogenous levels of these stress hormones during sepsis, (ii) the correlation between these hormones and monocyte mitochondrial and effector function in septic patients, and (iii) on the common use of these endocrine therapies in sepsis. The type and concentration of HKB, and the duration of incubation, were determined during an initial optimisation study. PBMCs were isolated from healthy volunteers and incubated with *Escherichia Coli* (EC) or *Staphylococcus Aureus* (SA), and/or three concentrations (correlating to reference values and levels found in sepsis and septic shock) of the above stress hormones. Mitochondrial and immune cell function of PBMCs, and monocytes in particular, were subsequently measured by flow cytometry.

3.2 Methodology

3.2.1 Study design

For *in vitro* PBMC stimulation, fresh PBMCs were isolated from healthy volunteers (six per study) by Ficoll gradient centrifugation of 10 mL samples of venous whole blood. Sodium heparin (1 IU/mL blood) (Fannin, Wellingborough, UK) was used to prevent clotting. PBMCs were initially plated on 96-well plates and incubated for three different durations (1, 6 and 24 hours) with three different concentrations of SA (10^6,

10^7, and 10^8 particles/mL) and EC (10^5, 10^6, and 10^7 particles/mL) (both InvivoGen, Toulouse, France). After incubation, cells were stained with LIVE/DEAD™ stain, pan-leukocyte and monocyte markers, HLA-DR, and mitochondrial dyes for measurement of MMP using TMRM, and mROS production using MitoSox. Samples were then analysed by flow cytometry. After optimisation, it was determined to continue further experiments with stress hormones with a 6-hour stimulation of cells with SA.

3.2.2 PBMC isolation

The 10 mL sample of venous blood was diluted in 20 mL PBS at room temperature (Gibco, Bleiswijk, The Netherlands) and layered carefully on top of 15 mL of Ficoll-paque PLUS (GE Healthcare, Chicago, IL, USA) in a 50 mL Falcon tube. This standardised medium is widely used for isolation of lymphocytes and monocytes from peripheral blood by a rapid density centrifugation procedure. Centrifugation for 30 minutes (1400 RPM, 20°C) without break separated the two layers into plasma, PBMCs, Ficoll-paque, granulocytes, and a bottom layer of red blood cells. The PBMC layer was subsequently aspirated, transferred into a clean 50 mL Falcon tube, and washed twice with 30 mL of PBS at room temperature by spinning samples for 8 minutes (1400 RPM, 20°C) with break.

After washing, PBMCs were diluted in 5 mL pre-warmed (37°C) Dulbecco's Modified Eagle Medium (DMEM) (Gibco). This contained 4.5 g/L D-glucose, L-Glutamine, and 25 mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES), but no phenol-red as this would disturb flow cytometry analyses. Media was supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and will from now on be referred to as complete cell culture media. Cell numbers and viability were assessed using Trypan Blue stain 0.4% (Invitrogen). A 1:1 mix by volume of cells and Trypan Blue was loaded onto Countess™ slides and read with the Countess II automatic cell counter (Invitrogen).

The cell suspension was subsequently diluted with complete cell culture media to a final concentration of $1x10^6$ cells/mL. A total of 100 uL of cell suspension was plated on sterile Nunclon™ 96-well plates (Thermo Fisher Scientific), resulting in 100.000 cells per well. Simultaneously, HKB (EC or SA) and/or stress hormones (adrenaline, noradrenaline, or hydrocortisone) were added to these wells as described in the next section. Cells with HKB only were incubated at 37°C with 5% CO_2 for 1, 6, and 24 hours during initial optimisation. A 6-hour incubation was then chosen for subsequent co-incubation with stress hormones.

3.2.3 Stimulation with HKB and hormones

During optimisation studies, the effects of both Gram-positive (SA) and Gram-negative (EC) bacteria on mitochondrial function (MMP and mROS production), and HLA-DR expression of PBMCs and monocytes were studied. Isolated PBMCs were incubated for 1, 6 and 24 hours with three different concentrations of SA and EC. Based on this initial optimisation study, I decided to continue with a 6-hour incubation of PBMCs at a concentration of 10^8 particles/mL of SA.

Subsequently, three different concentrations of adrenaline (Hameln, Gloucester, UK), noradrenaline (Aguettant, Lyon, France) and hydrocortisone were tested (Pfizer, Kent, UK). These three concentrations were selected based on plasma levels of catecholamines and glucocorticoids reported in the sepsis literature, and my own measurements in patient samples (Table 3). The lowest concentration represents the healthy normal range, whereas medium and high concentrations represent average and peak levels found in septic patients.

Table 3. Hormone concentrations based on literature and previous experiments. These were chosen based on levels found in healthy subjects and in septic patients.

Hormone	Low dose	Medium dose	High dose	Reference
Adrenaline	0.1 ng/mL	1 ng/mL	10 ng/mL	[66, 342, 417, 459]
Noradrenaline	1 ng/mL	10 ng/mL	100 ng/mL	[66, 342, 417, 459]
Hydrocortisone	0.1 ug/mL	1 ug/mL	10 ug/mL	[417, 492, 493]

3.2.4 Flow cytometry

3.2.4.1 Staining protocol

Flow cytometry was used to study the effects of HKB, and adrenaline, noradrenaline, and hydrocortisone on PBMCs, and monocytes in particular. Incubated cells were stained with viability markers and fluorescently labelled antibodies, loaded onto the LSR Fortessa™ X-20 Cell Analyzer (BD Biosciences), and analysed with FACS Diva™ Software (BD Biosciences). PBMCs were characterised by the pan-leukocyte marker CD45, whereas monocyte subsets were characterised by expression of HLA-DR, CD14, and CD16. Monocyte HLA-DR expression was used as a marker of antigen presentation. In addition, cells were incubated with TMRM or MitoSox for analysis of MMP and mROS production, respectively.

After incubation of cells with the two HKB and/or stress hormones, staining protocols for measurement of mROS and MMP were initiated (Table 4). Cells were incubated with 1 uL of a 1:10 dilution of LIVE/DEAD fixable Near-IR dead cell stain (Invitrogen) for 30 minutes at room temperature in the dark. After 15 minutes, cells were stained with 0.5 uL of CD14-APC (BioLegend), CD16-VioGreen (Miltenyi Biotec, Bergisch Gladbach, Germany), CD45-eFluor 450 (Invitrogen), and HLA-DR-BV711 (BioLegend) and left at room temperature in the dark for another 15 minutes.

Due to spectral overlap, MitoSox and TMRM (both Invitrogen) were added to the above cells in separate panels at the same time as all antibodies. For MitoSox, 1 uL of a 1:10 dilution was added to the cell suspension, and for TMRM, 5 uL of a 1:100

dilution of 50 uM. After incubation for another 15 minutes in the dark at room temperature, cells were transferred to 5 mL round bottom polystyrene tubes (Sarstedt) containing 300 uL of Hank's Balances Salt Solution (HBSS) (Gibco).

Table 4. Flow cytometry panels for TMRM and MitoSox.

			MitoSox panel		TMRM panel	
Laser	**Filter**	**Fluorochrome**	**Target**	**Clone**	**Target**	**Clone**
Violet (405 nm)	450/50 525/50 710/50	eFluor450 VioGreen BV711	CD45 CD16 HLA-DR	HI30 REA422 L243	CD45 CD16 HLA-DR	HI30 REA422 L243
Blue (488 nm)	582/15 710/50	PE PerCp-Cy5.5	 MitoSox		TMRM	
Red (640 nm)	670/30 780/60	APC Near-IR	CD14 LIVE/DEAD	HCD14	CD14 LIVE/DEAD	HCD14

3.2.4.2 Acquisition and gating strategy

Before each experiment, BD FACS Diva CS&T Research Beads (BD Biosciences) were run for calibration of the flow cytometer. Compensation was set using BD Anti-Mouse Ig CompBeads™ (BD Biosciences) for CD45, CD14, and HLA-DR. Healthy cells were used for compensation of CD16, LIVE/DEAD, TMRM, and MitoSox. To induce maximum fluorescence for the LIVE/DEAD stain, cells were heat-killed by incubation at 60°C for 15 minutes prior to staining. To induce maximum fluorescence for MitoSox, cells were incubated with 5 uL of 1:10 Antimycin A (10 uM) (Sigma-Aldrich) after full staining, and incubated at 37°C for 15 minutes.

In conjunction with the above-mentioned staining protocols, unstained control samples were run for every volunteer separately, and FMO controls were used to set gating strategies for CD45, HLA-DR, CD14, CD16, and LIVE/DEAD. Heat-killing of cells (60°C for 15 minutes) was used as positive control for LIVE/DEAD. For mitochondrial dyes, incubation with 5 uL of 1:10 FCCP (50 uM) (Sigma-Aldrich) prior

to staining with TMRM, and 5 uL of 1:10 Antimycin A, a mitochondrial ETC inhibitor (10 uM), after staining with MitoSox, were used as negative and positive controls, respectively.

For each sample, 2,000 HLA-DR^{+} cells were acquired. Cell populations were identified using sequential gating strategies in FlowJo Version 10 (Tree Star Inc.) (Figure 17). After the exclusion of debris and doublets on a SSC-A/FSC-A, and FSC-H/FSC-A plot respectively, PBMCs were gated based on pan-leukocyte CD45 expression. For these PBMCs, LIVE/DEAD^{-} live cells were selected. In live CD45^{+} PBMCs, the geometric means for TMRM, MitoSox, and HLA-DR were subsequently calculated. Following gating of CD45^{+} PBMCs, sub-populations of monocytes were identified based on expression of HLA-DR, CD14, and CD16. In these sub-populations, live cells were again selected based on negative LIVE/DEAD, and the geometric means for TMRM, MitoSox, and HLA-DR were subsequently calculated in these subsets.

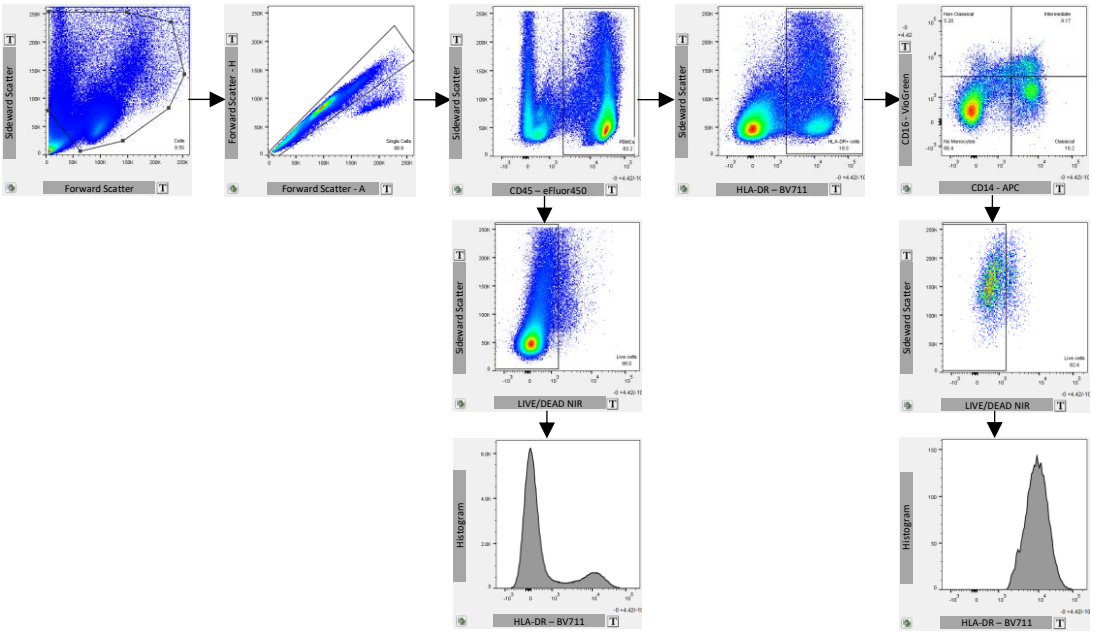

Figure 17. Gating strategy for TMRM, MitoSox, and HLA-DR in live PBMCs and monocytes.

3.2.5 Statistical analysis

Office Excel (Microsoft) was used for data organisation. Visualisation of data and statistical analysis were performed with GraphPad Prism Version 9 (GraphPad Software). Due to limited numbers of volunteers, data were visualised as median ± Interquartile Range (IQR). To study the effects of SA compared to unstimulated cells, Wilcoxon matched pairs signed rank test was used. For analysis of the effects of various concentrations of HKB, the effects of stress hormones on healthy cells, and the effects of stress hormones on SA treated cells, Friedman tests with subsequent uncorrected paired Dunn's tests were run. No corrections were applied due to the limited numbers of volunteers. A *P*-value <0.05 was considered significant.

3.3 Results

The effects of different concentrations of, and incubation times with, EC and SA, and the effect of 6 hour's incubation with SA and stress hormones on mitochondrial and immune function of PBMCs and monocytes were analysed using flow cytometry. Results for MMP (TMRM), mROS production (MitoSox), and HLA-DR expression are expressed as the geometric means of MFI (in Arbitrary Units (A.U.)) in live PBMCs and monocyte subsets.

3.3.1 HKB optimisation

The effects of three different concentrations of Gram-positive (SA) and Gram-negative (EC) bacteria on cell distribution, viability, mitochondrial function, and HLA-DR expression of total PBMCs and monocytes were studied during three time-points (1, 6 and 24 hours). Mitochondrial function (MMP and mROS production) and HLA-DR expression were measured in live cells only.

3.3.1.1 Viability

The viability of PBMCs and monocytes was calculated as percentage of LIVE/DEAD$^-$ cells (Supplementary Figure 2). While a 1-hour incubation with HKB did not result in significant differences in viable PBMCs or monocytes, a longer 6-hour incubation of PBMCs did (*P*=0.03), with decreased viability after SA (10^7) (*P*=0.0004) and SA (10^8) (*P*=0.02) stimulation. Overnight incubation of PBMCs with HKB resulted in changes in the viability of these cells (*P*=0.002). Incubation with both SA (10^7) and SA (10^8) resulted in decreased viability (*P*=0.04 and *P*=0.002, respectively).

For monocytes, a 6-hour incubation resulted in significant differences (*P*=0.008), with multiple comparisons showing a decreased viability after incubation with SA (10^7), SA (10^8), and EC (10^7) (*P*=0.0006, *P*=0.003, and *P*=0.03, respectively). Overnight 24-hour incubation also resulted in a significant difference between groups (*P*=0.0007), with a decrease in the number of viable monocytes incubated with SA (10^7), SA (10^8), and EC (10^6) (*P*=0.003, *P*=0.0003, and *P*=0.04, respectively).

3.3.1.2 Cell distribution

The number of total HLA-DR$^+$/CD14$^+$/CD16$^+$ monocytes was calculated as a percentage of total CD45$^+$ PBMCs (Supplementary Figure 3). After a 1-hour incubation, all but EC (10^7) (*P*=0.08) resulted in a significant decrease in the percentage of monocytes (*P*=0.03) compared to unstimulated cells. After 6 hours, most of these effects persisted (*P*=0.0008), with a significant decrease in monocytes after incubation with SA (10^6) (*P*=0.03), EC (10^6) (*P*=0.001), and EC (10^7) (*P*=0.009). After overnight 24-hour incubation, a significant decrease was again found (*P*=0.0005), specifically with SA (10^6) (*P*=0.003), EC (10^5) (*P*=0.03), and EC (10^6) (*P*=0.03).

3.3.1.3 Mitochondrial membrane potential

TMRM was used as an indicator of MMP (Supplementary Figure 4). After a 1-hour incubation of PBMCs with HKB, no differences were observed in the TMRM signal (*P*=0.51). After 6 hours, a significant increase was found in TMRM (*P*=0.04) for both SA (10^7) and SA (10^8) (*P*=0.005 and *P*=0.02, respectively). Stimulation for 24 hours did again not result in any significant changes in live PBMCs (*P*=0.16).

Even though especially SA seemed to decrease TMRM in monocytes at the various timepoints, incubation with HKB did not result in any significant differences in TMRM at 1 hour (*P*=0.30), 6 hours (*P*=0.39), or 24 hours (*P*=0.80),

3.3.1.4 Mitochondrial ROS production

PBMCs incubated with SA (10^8) for 1 hour seemed to elevate mROS, as measured by MitoSox signal, but none of the concentrations of HKB induced any significant changes at this timepoint (*P*=0.13) (Supplementary Figure 5). After 6 hours' incubation, differences were found between groups (*P*=0.005), with increased MitoSox expression in SA (10^7) (*P*=0.04) and SA (10^8) (*P*=0.0003). Overnight incubation resulted in a significant difference between groups (*P*=0.03), with increases in MitoSox seen in the two higher doses of SA (*P*=0.04 and *P*=0.0006) and EC (*P*=0.02 and *P*=0.05).

Specifically for monocytes, a 1-hour incubation with HKB did not result in significant differences between groups (*P*=0.12), even though especially SA (10^8) seemed to increase MitoSox in these cells. After 6 hours' incubation, MitoSox levels in monocytes increased (*P*=0.0008), specifically for higher doses of SA and EC (10^7) (all *P*=0.01). Incubation for 24 hours also showed significant differences between groups (*P*=0.03), with an increase in MitoSox with all HKB concentrations apart from EC (10^5) (*P*=0.06).

3.3.1.5 HLA-DR expression

HLA-DR expression on cells was used as a measure for antigen presenting capacity (Supplementary Figure 6). For PBMCs, no differences were found between groups after a 1-hour incubation with any of the HKB concentrations (*P*=0.41). However, after 6 hours' incubation, these did alter significantly (*P*=0.03), whereas after 24 hours of incubation, the differences had disappeared (*P*=0.13).

For monocytes specifically, no overall differences were detected between groups after a 1-hour incubation (*P*=0.30). After 6 and 24 hours of incubation, differences in HLA-DR expression between groups were found (*P*=0.01 and *P*=0.0008, respectively).

3.3.2 Adrenaline incubation

3.3.2.1 Viability and cell distribution

Isolated PBMCs were incubated with SA and three different concentrations of adrenaline for 6 hours. After incubation, LIVE/DEAD stain was used to distinguish live from dead cells in PBMCs, and in monocyte sub-populations (Figure 18). For PBMCs, viability remained high across all treatment conditions. For non-classical monocytes, median viability was decreased after SA incubation (*P*=0.03), but this effect was not seen for intermediate or classical monocytes (*P*=0.31 and *P*>0.99, respectively). No impact was seen with adrenaline co-incubation under any condition.

Monocyte sub-populations were determined based on surface expression of HLA-DR, and CD14 and CD16 antibodies. Total monocytes are expressed as percentage of $CD45^+$ PBMCs, whereas monocyte sub-populations as percentage of total monocytes (Figure 18). No significant changes were found in the total number of monocytes after treatment with either SA, adrenaline, or both. For non-classical and intermediate monocytes, a significant increase in the number of these cells was observed after incubation with SA, whereas the same treatment resulted in a significant decrease in

classical monocytes (*P*=0.03 for all). Adrenaline did not further affect the distribution of these monocyte subsets.

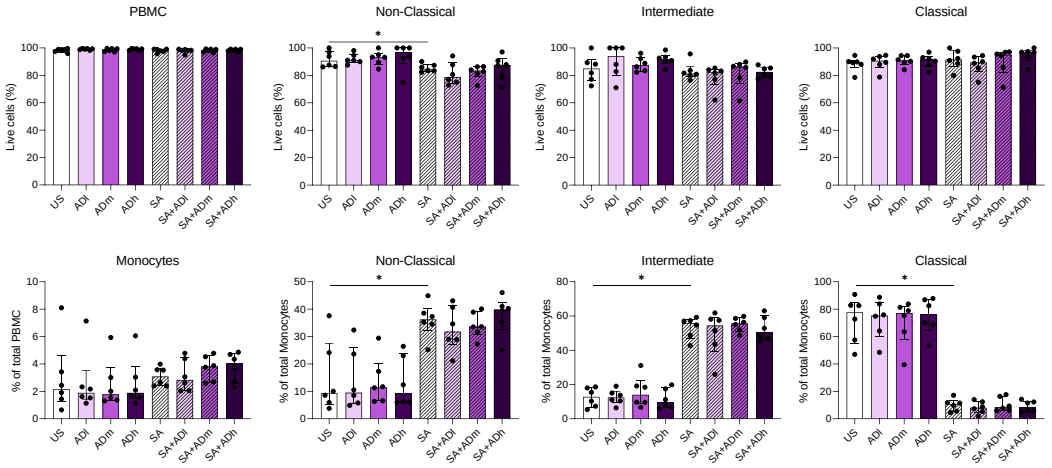

Figure 18. Viability and distribution of cells after incubation with SA and/or adrenaline. Isolated PBMCs from healthy volunteers (*n*=6) were incubated for 6 hours with SA and/or three different concentrations of adrenaline. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocyte subsets (being Non-Classical, Intermediate and Classical monocytes). The number of live (LIVE/DEAD$^-$) PBMCs and monocytes are expressed as percentage of the total amount of these cells. The number of monocytes is expressed as percentage of the total amount of PBMCs in the sample. The number of monocyte subsets is expressed as the total amount of monocytes in the sample. Data presented as median ± IQR. US: Unstimulated; SA: S. Aureus; ADl: Adrenaline low dose (0.1 ng/mL); ADm: Adrenaline medium dose (1 ng/mL); ADh: Adrenaline high dose (10 ng/mL). **P*<0.05.

3.3.2.2 Mitochondrial markers and HLA-DR

Even though viability after 6 hours of incubation was good in PBMCs and monocyte sub-populations, subsequent analysis of TMRM, MitoSox, and HLA-DR was performed in live cells only, to limit the effects of dead cells in the samples. MMP was measured by flow cytometry as the geometric mean of TMRM MFI (Figure 19).

SA stimulation did not affect the TMRM signal of PBMCs, nor of any of the monocytes. Although differences were found between healthy non-classical monocytes treated with adrenaline (*P*=0.04), multiple comparisons did not detect individual significant

changes. Incubation of PBMCs with SA and adrenaline resulted in differences between groups (*P*=0.04), with a decrease in the TMRM signal with a low dose (*P*=0.01). Although adrenaline seemed to decrease TMRM in non-classical monocytes, differences between groups did not reach significance (*P*=0.06).

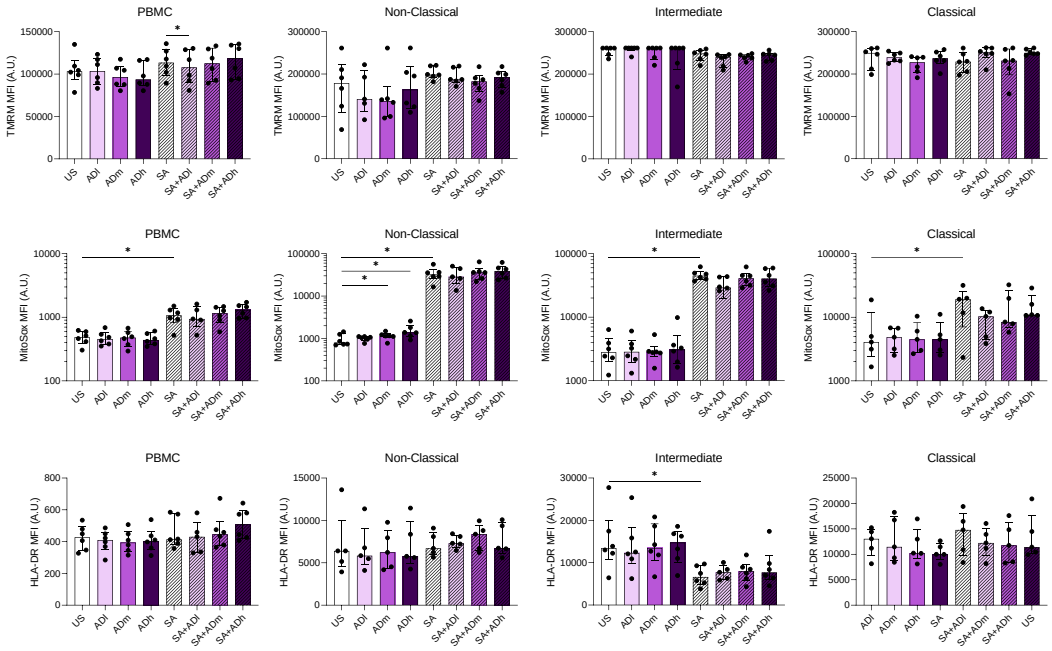

Figure 19. TMRM, MitoSox, and HLA-DR signal in live cells after incubation with SA and/or adrenaline. Isolated PBMCs from healthy volunteers (*n*=6) were incubated for 6 hours with SA and/or three different concentrations of adrenaline. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocyte subsets (being Non-Classical, Intermediate and Classical monocytes). TMRM (measuring MMP), MitoSox (measuring mROS) and HLA-DR signal was measured in live (LIVE/DEAD^{-}) monocytes only. Data presented as median ± IQR. A log-scale was used for presentation of MitoSox. MFI: Mean Fluoresce Intensity; A.U.: Arbitrary Units; US: Unstimulated; SA: S. Aureus; ADl: Adrenaline low dose (0.1 ng/mL); ADm: Adrenaline medium dose (1 ng/mL); ADh: Adrenaline high dose (10 ng/mL). **P*<0.05; ***P*<0.01.

mROS production was measured by flow cytometry as the geometric mean of MitoSox MFI (Figure 19). SA stimulation increased the MitoSox signal of all cells (*P*=0.03). In healthy non-classical monocytes, the addition of adrenaline resulted in a dose-dependent change in MitoSox (*P*=0.04), with both a medium (*P*=0.03) and high dose

(*P*=0.04) resulting in an increased signal. The visual decrease in mROS in SA treated intermediate monocytes did not reach significance (*P*=0.07). No other significant changes in MitoSox after adrenaline addition were observed.

HLA-DR expression is visualised as the geometric mean of MFI (in A.U.) (Figure 19). Stimulation of cells with SA only resulted in a significant decrease in HLA-DR on intermediate monocytes (*P*=0.03). Although noradrenaline seemed to increase HLA-DR in SA treated non-classical and intermediate monocytes, and decrease in classical monocytes, these changes did not reach significance (*P*=0.37, *P*=0.09, and *P*=0.09, respectively).

3.3.3 Noradrenaline incubation

3.3.3.1 Viability and cell distribution

Viability of PBMCs and monocyte sub-populations after noradrenaline incubation was assessed by LIVE/DEAD near-IR (Figure 20). Viability of cells was affected by SA treatment in non-classical monocytes only (*P*=0.03). The addition of noradrenaline to cells did not result in any significant changes for PBMCs, nor intermediate and classical monocytes. However, in SA treated non-classical monocytes, a high concentration of noradrenaline caused a drop in viability (Friedman: *P*=0.03; Dunn's: *P*=0.004).

Monocyte sub-populations were determined based on surface expression of HLA-DR, and CD14 and CD16 antibodies, and expressed as percentage of total $CD45^+$ PBMCs (Figure 20). The total number of monocytes in the sample did not change upon addition of SA (*P*=0.44), but the distribution of sub-populations did. A significant increase in the percentage of non-classical and intermediate monocytes, and a decrease in the percentage of classical monocytes was observed (*P*=0.03 for all).

The addition of noradrenaline to SA treated non-classical monocytes indicated differences between groups (*P*=0.02), but subsequent multiple comparisons were not significant. SA treated classical monocytes did show differences after noradrenaline incubation (*P*=0.04). In these cells, a high concentration of noradrenaline resulted in decreased numbers of classical monocytes (*P*=0.04).

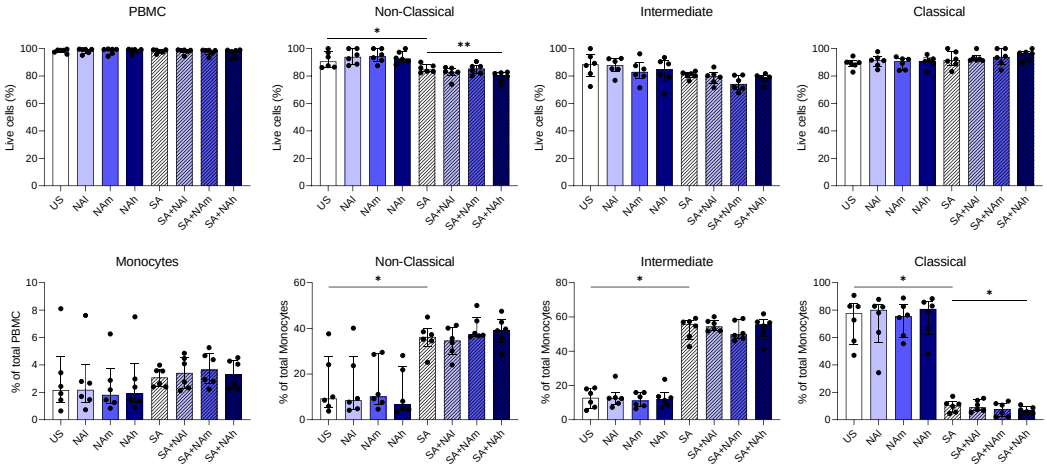

Figure 20. Viability and distribution of cells after incubation with SA and/or noradrenaline. Isolated PBMCs from healthy volunteers (*n*=6) were incubated for 6 hours with SA and/or three different concentrations of noradrenaline. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocyte subsets (being Non-Classical, Intermediate and Classical monocytes). The number of live (LIVE/DEAD$^-$) PBMCs and monocytes are expressed as percentage of the total amount of these cells. The number of monocytes is expressed as percentage of the total amount of PBMCs in the sample. The number of monocyte subsets is expressed as the total amount of monocytes in the sample. Data presented as median + IQR. US: Unstimulated; SA: S. Aureus; NAl: Noradrenaline low dose (1 ng/mL); NAm: noradrenaline medium dose (10 ng/mL); NAh: Noradrenaline high dose (100 ng/mL). **P*<0.05; ***P*<0.01.

3.3.3.2 Mitochondrial markers and HLA-DR

or PBMCs and all monocyte sub-populations, incubation with SA did not affect TMRM signal (Figure 21). Incubation of healthy and SA treated cells with noradrenaline neither resulted in any significant changes in TMRM.

The geometric mean of MitoSox MFI was used to measure mROS production in live PBMCs and monocyte sub-populations. The addition of SA increased MitoSox signal significantly in all cell populations (*P*=0.03 for all). Although noradrenaline in all three concentrations seemed to increase MitoSox in non-classical monocytes, this effect did not reach significance (*P*=0.09). Similarly, the visual decrease in SA treated classical monocytes also did not reach significance (*P*=0.57).

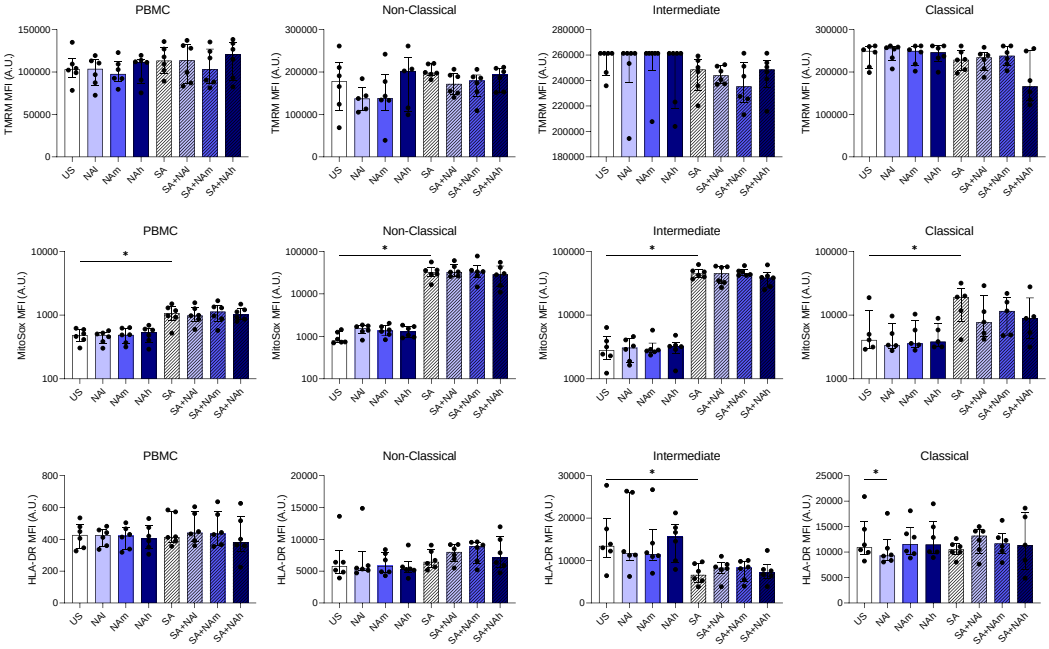

Figure 21. TMRM, MitoSox, and HLA-DR signal in live cells after incubation with SA and/or noradrenaline. Isolated PBMCs from healthy volunteers (*n*=6) were incubated for 6 hours with SA and/or three different concentrations of noradrenaline. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocyte subsets (being Non-Classical, Intermediate and Classical monocytes). TMRM (measuring MMP), MitoSox (measuring mROS) and HLA-DR signal was measured in live (LIVE/DEAD^{-}) monocytes only. Data presented as median + IQR. A log-scale was used for presentation of MitoSox. MFI: Mean Fluoresce Intensity; A.U.: Arbitrary Units; US: Unstimulated; SA: S. Aureus; NAl: Noradrenaline low dose (1 ng/mL); NAm: noradrenaline medium dose (10 ng/mL); NAh: Noradrenaline high dose (100 ng/mL). *P<0.05.

HLA-DR expression was measured as the geometric mean of HLA-DR-APC MFI for PBMCs, and separately for the three monocyte sub-populations (Figure 21).

Incubation with SA only affected intermediate monocytes, with a significant repression of HLA-DR expression (*P*=0.03). Although noradrenaline seemed to increase HLA-DR in SA treated non-classical and intermediate monocytes, these effects did not reach significance (*P*=0.38 and *P*=0.16, respectively). Contrary, a drop in HLA-DR was observed in healthy classical monocytes (Friedman: *P*=0.03; Dunn's: *P*=0.03).

3.3.4 Hydrocortisone incubation

3.3.4.1 Viability and cell distribution

LIVE/DEAD stain was used to distinguish live from dead cells in PBMCs, and in monocyte sub-populations (Figure 22). Incubation with SA did not affect viability in any of the cells, neither did hydrocortisone addition to healthy or SA treated cells.

The total number of monocytes in the PBMC population, and subsequent distribution of sub-populations of monocytes, were calculated and are visualised in Figure 22. Incubation of cells with SA significantly affected the distribution of monocytes, with an increase in non-classical and intermediate, but decrease in classical cells (*P*=0.03 for all). In healthy PBMCs, a medium and high dose of hydrocortisone both increased the number of monocytes present (Friedman: *P*=0.04; Dunn's: *P*=0.01 for both). Although a high dose of hydrocortisone seemed to increase the number of healthy non-classical monocytes, no significant effects of hydrocortisone were found for any of the healthy nor SA treated monocyte sub-populations.

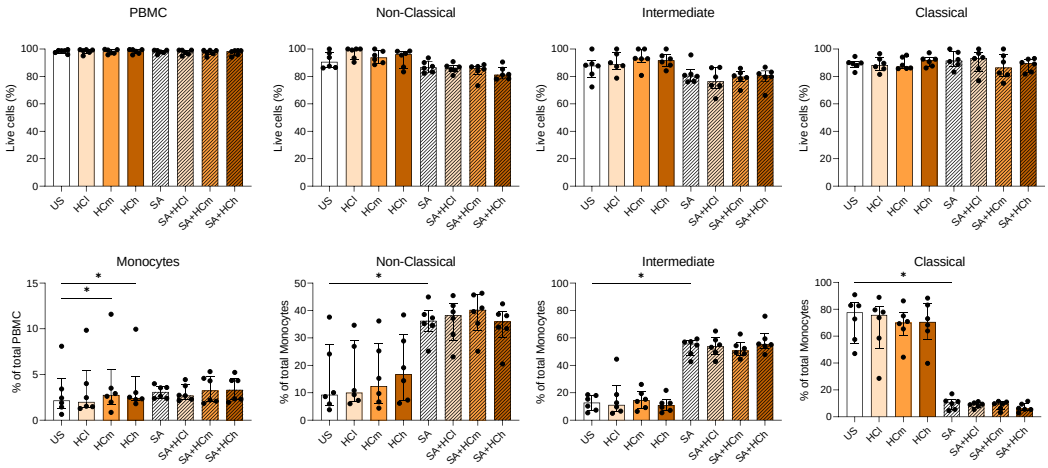

Figure 22. Viability and distribution of cells after incubation with SA and/or hydrocortisone. Isolated PBMCs from healthy volunteers (*n*=6) were incubated for 6 hours with SA and/or three different concentrations of hydrocortisone. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocyte subsets (being Non-Classical, Intermediate and Classical monocytes). The number of live (LIVE/DEAD^{-}) PBMCs and monocytes are expressed as percentage of the total amount of these cells. The number of monocytes is expressed as percentage of the total amount of PBMCs in the sample. The number of monocyte subsets is expressed as the total amount of monocytes in the sample. Data presented as median ± IQR. US: Unstimulated; SA: S. Aureus; HCl: Hydrocortisone low dose (0.1 ug/mL); HCm: Hydrocortisone medium dose (1 ug/mL); HCh: Hydrocortisone high dose (10 ug/mL). *P<0.05.

3.3.4.2 Mitochondrial markers and HLA-DR

The addition of SA did not result changes in TMRM for any of the cells. Hydrocortisone did however significantly affect TMRM signal in healthy PBMCs (P=0.009), with a decrease in TMRM with a low (P=0.03) and medium dose (P=0.002). A similar effect of hydrocortisone happened in SA treated PBMCs (P=0.04), with again a low (P=0.04) and high dose (P=0.007) of hydrocortisone decreasing the TMRM signal.

MitoSox was used as a measure of mROS production in live PBMCs and monocyte subsets. The addition of SA resulted in a significant increase in MitoSox in PBMCs and all monocyte subsets (P=0.03 for all). Although hydrocortisone seemed to

increase mROS in both healthy and SA stimulated PBMCs, these effects did not reach significance (*P*=0.06 for both). However, in healthy non-classical monocytes, these additions did result in a significant increase in mROS (Friedman: *P*=0.006; Dunn's: *P*=0.01 for a medium and *P*=0.002 for a high dose). On the contrary, hydrocortisone changed mROS in healthy intermediate monocytes (*P*<0.0001), with a medium (*P*=0.04) and high dose (*P*=0.0003) both causing a decrease.

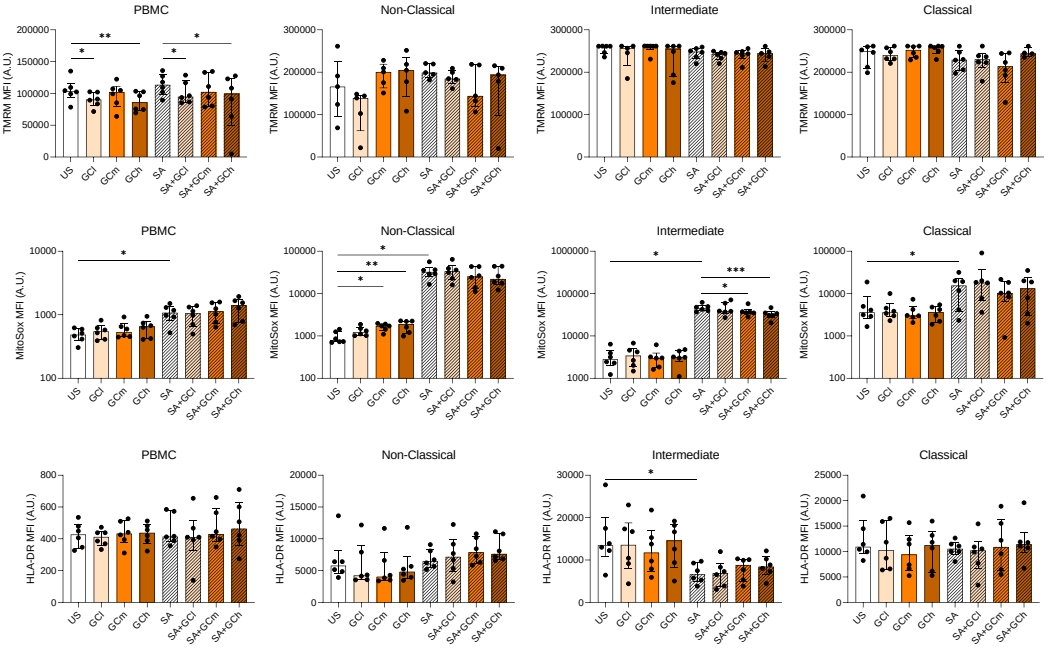

Figure 23. TMRM, MitoSox, and HLA-DR signal in live cells after incubation with SA and/or hydrocortisone. Isolated PBMCs from healthy volunteers (*n*=6) were incubated for 6 hours with SA and/or three different concentrations of hydrocortisone. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocyte subsets (being Non-Classical, Intermediate and Classical monocytes). TMRM (measuring MMP), MitoSox (measuring mROS) and HLA-DR signal was measured in live (LIVE/DEAD$^-$) monocytes only. Data presented as median + IQR. A log-scale was used for presentation of MitoSox. US: Unstimulated; SA: S. Aureus; HCl: Hydrocortisone low dose (0.1 ug/mL); HCm: Hydrocortisone medium dose (1 ug/mL); HCh: Hydrocortisone high dose (10 ug/mL). **P*<0.05; ***P*<0.01; ****P*<0.001.

HLA-DR as a measure for antigen presentation capacity was measured in live PBMCs and monocyte-subsets (Figure 23). Incubation of cells with SA only affected HLA-DR

expression in intermediate monocytes, causing a decrease (*P*=0.03). Hydrocortisone addition to healthy cells did not have any significant effects, despite the seemingly decrease in non-classical monocytes (*P*=0.11). The visual increase in SA treated non-classical and intermediate monocytes caused by hydrocortisone also did not reach significance (*P*=0.07 and *P*=0.19, respectively).

3.4 Discussion

Viability of PBMCs and the monocyte subsets were increasingly affected by both a longer duration of incubation (from 1 hour to 24 hours) with HKB, and also by higher doses of SA and EC. SA had a greater impact on viability compared to EC. At all timepoints, some of the concentrations of both SA and EC decreased the number of total monocytes. Incubation with SA or EC had a variable effect on MMP (measured by TMRM) in PBMCs, and this varied with both incubation duration and concentration. On the other hand, both HKB produced a time- and dose-dependent increase in mROS (measured by MitoSox), both in PBMCs and in all monocyte subsets, though the most prominent increase was seen with SA. Whereas SA mainly decreased HLA-DR expression on monocytes after 6 and 24 hours' incubation, EC consistently increased HLA-DR expression on monocytes.

Subsequent 6-hour incubation of cells with SA and/or adrenaline did not result in any changes in viability or cell distribution, nor in changes to mitochondrial markers or HLA-DR expression in intermediate and classical monocytes. However, in SA treated PBMCs, a low dose of adrenaline caused a drop in TMRM signal. In addition, both medium and high doses of adrenaline increased MitoSox in healthy non-classical monocytes.

The highest dose of noradrenaline decreased both the viability of SA treated classical monocytes, and the number of these cells. However, TMRM nor MitoSox were

affected by noradrenaline co-incubation. The only effect observed was a decrease in HLA-DR expression of healthy classical monocytes with a low dose of noradrenaline.

The two highest doses of hydrocortisone increased the number of monocytes in healthy PBMCs. These doses also increased mROS in healthy non-classical monocytes, but decreased mROS in SA treated intermediate monocytes. A low and high dose decreased MMP in both healthy and SA treated PBMCs. No effects were observed in HLA-DR expression after co-incubation with hydrocortisone.

3.4.1 Differential effects of HKB

The variable effects on viability of SA and EC may relate to the higher concentrations of SA being used, though these doses were recommended in the supplier's product information for such *in vitro* studies (10^6-10^8/ml for SA and 10^5-10^7/ml for EC). Although I tried to reduce the effects of dead cells by excluding them from flow cytometry analysis, their presence in cell culture could have affected neighbouring live cells. Nonetheless, the viability in most samples exceeded the minimum suggested viability for PBMCs of 89% [494].

In septic patients, low monocyte counts are associated with an increased risk of mortality and an increased incidence of bacteraemia and organ dysfunction [495]. A change in blood monocyte composition, with an increase in $CD14^{dim}/CD16^{+}$ monocytes, has also been found in sepsis [34]. However, the decrease in the number of monocytes in my current study could also be explained by HKB-induced changes in surface molecule expression and subsequent adhesion to cell culture plates. These results are therefore not necessarily representative of the total number of monocytes present in cell culture.

The differential effects of SA and EC on mitochondrial markers and HLA-DR expression could potentially be explained by their distinct characteristics and the

ensuing host response. Infection with Gram-negative and Gram-positive bacteria induces a differential disease presentation, with differences in host signalling pathways, cytokine release, and clinical outcomes [496]. Bacteria cause most sepsis cases, with an increasing proportion of gram-positive rather than gram-negative sepsis. In addition, mortality due to gram-positive infection is higher than from gram-negative sepsis [425]. This relates in part to activation of different host PRRs, with Gram-negative bacterial components such as LPS having a TLR4 dominant signal, and Gram-positive bacterial components such as lipoteichoic acid being mainly recognised by TLR2.

These TLRs recruit different adaptor proteins and subsequently activate different transcription factors, including Nuclear Factor Kappa B (NF-kB), AP-1, and INF Response Factor-3 (IRF-3) [497]. TLR4 signalling involves both Myeloid Differentiation Primary Response 88 (MyD88) dependent and independent pathways. TLR2 can form heterodimers with other TLRs such as TLR1 and TLR6, activating NF-kB and AP-1 via the MyD88 dependent pathway. Cytokine profiles differ amongst these two classes of bacteria. Gram-negative infection is, for example, associated with higher levels of TNF-α, IL-6, and IL-10, and lower levels of IFN-γ [498, 499].

In vitro exposure of monocytes to bacteria or bacterial products is associated with upregulation of monocyte HLA-DR [500]. However, downregulation of MHC class II transcription is a common strategy used by certain pathogens to circumvent immune recognition. This may be mediated either at transcriptional [501] or post-transcriptional [502] levels, depending on the specific pathogen, and may explain conflicting reports on levels of monocyte HLA-DR expression in sepsis [503, 504].

3.4.2 Stress hormone-induced effects

3.4.2.1 Adrenaline

3.4.2.1.1 Mitochondrial function

MMP is a key indicator of mitochondrial activity and a driving force behind ATP formation by OXPHOS. Catecholamines, including adrenaline, normally affect mitochondrial metabolism by inducing lipolysis and glycogenolysis, mobilising energy substrates for oxidation and conversion into ATP. For example, adrenaline rapidly increased mitochondrial activity and ATP in healthy rat hepatocytes, though longer term effects are less well known [190]. In my study, a low dose of adrenaline however decreased MMP in SA-treated PBMCs.

These results are more in line with a recent study in which 24-hour adrenaline incubation decreased both basal and maximal O_2 consumption, and the maximal rate of glycolysis in LPS-stimulated monocytes and whole blood, although PBMCs were not studied [193]. However, when monocytes were incubated with adrenaline and subsequently rested for 5 days, the addition of LPS increased basal and maximal O_2 consumption and glycolysis in these cells, indicating that adrenaline-induced effects depend on previous exposure and environmental factors.

Another potential mechanism explaining the adrenaline-induced decrease in MMP is via its effect on thermogenesis and uncoupling. This could divert the use of O_2 away from ATP production towards heat generation. Thermogenesis is induced via β_3-AR stimulation and downstream regulation of UCPs [505, 506]. β_3-ARs have been identified on both lymphocytes and macrophages [507]. Potential adrenaline-induced uncoupling could also attenuate mROS production and protect against cellular damage [508]. However, rather than a decrease, I found increased mROS with medium and high doses of adrenaline in healthy non-classical monocytes.

3.4.2.1.2 Immune function

To my knowledge, no other studies have investigated the effects of adrenaline on HLA-DR expression. I did not find any significant effects of adrenaline on HLA-DR expression of PBMCs and monocytes. Previous studies suggest anti-inflammatory cytokine IL-10 to be a depressant of monocyte HLA-DR expression [509, 510]. However, IL-10 release was not assessed in the current study so no conclusions can be drawn on this potential underlying mechanism.

Adrenaline is known to inhibit antigen presentation via β_2-ARs in Langerhans- and epidermal cells *in vitro* and in an *in vivo* mouse model [511]. In another mouse model, β_2-AR agonists reduced the ability of DCs to cross-present protein antigen to $CD8^+$ T-cells, whilst exogenous MHC Class I peptide presentation was preserved [512]. The opposite was observed in B-cells, where β_2-AR signalling increased the ability to present antigen and activate $CD4^+$ T-cell through co-stimulatory molecules CD80/CD86 [513, 514].

Other immunoregulatory effects of adrenaline and AR activation on innate immune cells, and monocytes and macrophages specifically, have been summarised in various reviews [68, 515, 516]. In summary, the effects of adrenaline are usually anti-inflammatory and immunosuppressive, but there are some inconsistencies in the literature. *In vitro*, *ex vivo*, and *in vivo* endotoxin studies have shown immunosuppressive effects of adrenaline in stimulated cells, with decreased production of pro-inflammatory cytokines and chemokines, suppression of β_2-integrin (C11b) on monocytes, and increased production of the anti-inflammatory cytokine IL-10 [332, 343-345, 347, 517-519]. Most of these effects were mediated via β_2-ARs and activation of the cAMP-PKA signalling pathway, and exerted at a posttranscriptional level [332, 346, 517].

By contrast, immune-enhancing effects of adrenaline have also been reported. Adrenaline for example regulated motility and expansion of haematopoietic progenitor

cells, and induced proliferation and mobilization of bone marrow mononuclear cells in an *in vivo* mouse model [520]. More specifically, in monocytes, adrenaline, or specific β_2-AR activation by terbutaline, enhanced production and mRNA expression of IL-8 and the anti-inflammatory IL-10 [332], likely mediated via cAMP formation [521].

These discrepancies could potentially be explained by alternative signalling mechanisms induced by β_2-AR activation, or differential effects of α- and β-AR signalling. Alternative signalling mechanisms induced by β_2-AR activation in non-immune cells have been identified, such as a switch from cAMP-PKA to the MAPK pathway. This alternative signalling may explain inconsistent findings regarding immune function in the literature and within the current study [522].

Additionally, whereas stimulation of the β-AR inhibits ROS production, phagocytosis, and the production of pro-inflammatory cytokines by monocytes [523-525], α-AR stimulation in LPS-stimulated macrophages and monocytes increased TNF-α and IL-1β expression [526]. Protein kinase C and MAPK signalling were identified as responsible downstream pathways for these pro-inflammatory effects. These observations suggest the differential roles of catecholamines on macrophages may depend on the AR subtype and the subsequent pathways activated, and thus dependent on cell type and concentration and duration of exposure [515].

To study these discrepancies in more detail, van der Heijden et al. (2020) studied differences in adrenaline-mediated effects of untrained and trained monocytes. Stimulation of whole blood and monocytes with LPS and adrenaline for 24 hours resulted in the attenuated release of TNF-α and IL-6. In contrast, after 5 days of rest, adrenaline-trained monocytes increased IL-6 and TNF-α production on exposure to LPS. These effects appeared to be mediated via the β-AR-cAMP-PKA pathway [193].

3.4.2.2 Noradrenaline

3.4.2.2.1 Mitochondrial function

As with adrenaline, noradrenaline generally affects mitochondrial metabolism by mobilising energy substrates from reserves to augment their availability for oxidation by mitochondria. Noradrenaline also increases lipolysis and glycogenolysis by interacting with both β_1- and β_2-ARs, suppressing glycolysis, and inducing insulin resistance [225].

Noradrenaline rapidly increased the RCR, rate of O_2 consumption, and rate of ATP formation in rat liver mitochondria isolated from post-burn injury rats, but these effects could be partially blocked with both α- and β-AR blockers [190]. These results are not in line with my findings where TMRM was not affected by noradrenaline. In another study, noradrenaline inhibited O_2 consumption at physiological doses in quiescent PBMCs [195]. β-AR antagonists, but not α-AR antagonists, reversed this inhibition. By contrast, α-AR antagonists, but not β-AR antagonists, reversed these noradrenaline effects in mitogen-activated PBMCs. These data suggest that the effects of noradrenaline on PBMC mitochondria depend on the activation state of the cells and AR signalling.

Van der Heijden et al. (2020) proposed monocyte training as an explanation for these differential effects. Incubation of primary monocytes with noradrenaline and LPS for 24 hours decreased basal and maximal O_2 consumption and glycolysis. By contrast, noradrenaline-trained monocytes increased both basal and maximal O_2 consumption, and glycolysis after 6 days following LPS exposure [193].

Acute (30 minutes) exposure of T-lymphocytes to noradrenaline had no effect on baseline O_2 consumption nor ROS production [527]. However, after 96 hours of incubation, decreased reserve and maximal respiratory capacity were observed, without affecting mitochondrial ATP levels. This suggests bioenergetic dysfunction

and a potential mitochondrial source of increased ROS. These effects appeared to be AR dependent as specific AR-antagonists could reverse the increase in ROS. A build-up of cAMP in response to noradrenaline could alter mPTP function, affect mitochondrial polarisation and, subsequently, ROS production.

However, I did not find any significant effects of noradrenaline on mROS. A recent study demonstrated that noradrenaline suppressed both glycolysis and the spare respiratory capacity of primary monocytes stimulated by LPS [196]. Pre-incubation of monocytes with noradrenaline for one hour decreased Phorbol Myristol Acetate (PMA)-induced ROS production by these cells while noradrenaline also caused a dose-dependent attenuation in basal neutrophilic ROS production *in vitro*. However, it must be noted that the doses of noradrenaline used in this study exceed the maximum reported levels that cells are normally exposed to in sepsis.

3.4.2.2.2 Immune function

Noradrenaline directly modulates both innate and adaptive immune cell function *in vitro* and *in vivo* by binding to α- and β-ARs expressed on these cells. Noradrenaline has predominant α-AR affinity, although it does stimulate β-ARs to a certain degree. However, like adrenaline, data on the direction of noradrenaline effects on immune cells are inconsistent. Most findings suggest predominantly immunosuppressive effects, whereas others indicate pro-inflammatory effects.

Specific effects of noradrenaline on HLA-DR expression are less well studied. I found a drop in HLA-DR on healthy classical monocytes with a low dose of noradrenaline. As with adrenaline, a potential mechanism underlying noradrenaline effects on HLA-DR expression is the anti-inflammatory cytokine, IL-10, a known depressant of monocyte HLA-DR [509, 510, 528-530]. However, *in vivo* and *in vitro* studies offer conflicting evidence. Downregulation of HLA-DR on classical monocytes was observed in cardiogenic shock patients and correlated with the noradrenaline dose

used for treatment [458]. On the other hand, *in vitro* experiments in monocytes and whole blood found no effect of noradrenaline on HLA-DR expression [459, 460].

Other noradrenaline-mediated effects on immune regulation are reported in various *in vitro* and *in vivo* animal studies, and in clinical studies. Noradrenaline-mediated effects are AR-dependent, resulting in increased TNF-α, IL-1β, and IL-6 mediated via the NF-kB pathway [329, 330]. However, Yu et al. [331] demonstrated anti-inflammatory effects following α-AR stimulation by noradrenaline. Notwithstanding these effects on the α-AR, the effects of noradrenaline appear to be mainly β-AR dependent, working via enhanced intracellular cAMP and the induction of PKA as an inhibitor of NF-kB, ultimately resulting in reduced expression of pro-inflammatory cytokines [330, 336].

This β-AR pathway enhanced synthesis of IL-10 and attenuated the release of TNF-α, IL-6, and IL-8 [332]. Addition of β-blockers, in particular β_2-AR antagonists, diminished this noradrenaline-induced effect [339, 340, 345, 531]. Other immunosuppressive effects of noradrenaline acting via the β_2-AR include diminished NK cell cytotoxicity and downregulation of IL-2 by Th1 cells, skewing the immune response from a Th1 to a Th2 phenotype [333, 334]. Noradrenaline also induces an immunosuppressive phenotype in neutrophils and promotes bacterial growth *in vitro* for both Gram-positive and Gram-negative bacteria [335, 337]. This results in increased susceptibility to infections [341]. In an *in vivo* caecal ligation mouse model, noradrenaline increased bacterial dissemination to other tissues [196].

Despite the plethora of *in vitro* and animal studies, limited clinical studies have investigated the immunologic effects of noradrenaline. Blocking the β-AR in paediatric patients with high catecholamine levels secondary to burn injury was associated with diminished secondary infections [532]. In septic patients, higher dose noradrenaline infusion correlated with a pro-inflammatory cytokine balance, whereas β-blocker use was associated with an overall anti-inflammatory balance [196]. In a human

endotoxemia model, a specific β_1-AR agonist did not affect cytokine release, suggesting that the anti-inflammatory effects of noradrenaline are mediated by other β-ARs [346]. In another human endotoxemia model, noradrenaline enhanced IL-10 production and attenuated release of the pro-inflammatory IP-10 [196].

Another potential explanation of this discrepancy in the immunomodulatory effects of noradrenaline is immune cell training. Whole blood and primary monocytes incubated with LPS and noradrenaline for 24 hours resulted in dose-dependent decreases in both TNF-α and IL-6 production. By contrast, after 5 days of rest following noradrenaline stimulation, LPS stimulation increased pro-inflammatory cytokine production. This training was established via the β-AR-cAMP pathway [193].

3.4.2.3 Hydrocortisone

3.4.2.3.1 Mitochondrial function

Glucocorticoids stimulate mitochondrial metabolism by mobilising energy substrates [225]. As mitochondria contain GRs for mitochondrially-encoded OXPHOS genes, gene expression and mitochondrial energy metabolism can be directly regulated [219, 462, 463]. In addition to these translational effects, glucocorticoids can also trigger activation of kinase signalling pathways, including P13K, AKT, MAPK, and other downstream intracellular signalling pathways [200].

It does appear that hydrocortisone and other glucocorticoids also affect mitochondria in a time- and dose-dependent manner. Short-term, limited exposure is generally stimulatory and increases mitochondrial capacity in liver, muscle, kidney, and cortical neuronal cells. This is accomplished through activation of respiratory chain components, enhancement of mitochondrial and nuclear gene expression, increased MMP, prevention of apoptosis and cell death, and by increasing mitochondrial biogenesis and mtDNA content [211, 213-217, 533, 534].

Additional effects of glucocorticoids include regulation of mitochondrial Ca^{2+} content and thermoregulation. In skeletal muscle and cortical neuronal mitochondria, glucocorticoids improved respiration through Ca^{2+} accumulation [211, 218]. Activation of both GRs and MRs can inhibit the uncoupling proteins UCP1 and UCP3 in BAT involved in thermoregulation, preventing dissipation of the proton gradient and maintaining ATP generation [231]. By contrast, restraint stress in an *in vivo* mouse model with a subsequent increase in corticosterone, as well as *in vitro* dexamethasone and corticosterone treatment, directly increased macrophage UCP2 expression [535]. Chronic treatment with corticosterone in an *in vivo* rat model also resulted in uncoupling [536].

Although the PBMCs in my study were only exposed to hydrocortisone for 6 hours, I observed the opposite of the acute short-term effects on MMP described above. Similarly, Hunter et al (2016) also found biphasic effects on the rat hippocampus [208]. Acute stress decreased mtRNA expression of Complex I and V genes, while chronic stress induced elevated expression of Complex I genes. In an *in vivo* endotoxaemia model, circulating leukocytes lowered protein translation of mitochondrial complex elements with increased plasma cortisol concentrations [537]. Acute *in vivo* treatment of rats with corticosterone decreased respiration and rates of ATP synthesis [536].

My results more resemble the effects of long-term exposure to glucocorticoids which causes respiratory chain dysfunction through inhibition of Complex I, IV, and V activity, decreased ATP production, increased ROS generation, mitochondrial structural abnormalities, abnormal mitochondria, biogenesis, decreased MMP, and increased sensitivity to cell death in brain, muscle, lymphoma, and kidney cells [199, 224, 225, 228, 229, 534]. Long-term exposure also inhibited Ca^{2+} influx in skeletal muscle cells and cortical neurons [211, 230].

Although markers of apoptosis were not specifically measured, the fall in MMP induced by hydrocortisone could potentially be explained by the induction of

apoptosis. Glucocorticoids are potent inducers of apoptosis in many cell types and tissues, including macrophages, lymphocytes, and other cells of the innate and adaptive immune system [538-540]. The classical mechanism of glucocorticoid-induced apoptosis involves the activation of the intrinsic pathway which involves mitochondria [541]. Activation of pro-apoptotic proteins Bax/Bak disrupts the MMP by opening the mPTP with release of cytochrome C and other apoptogenic proteins, leading to activation of caspase 9, caspase 3, and subsequent apoptotic cell death [542]. However, there may be a biphasic response; short-term exposure to glucocorticoids induced mitochondrial translocation of its receptor, increasing B-cell Lymphoma 2 (Bcl-2) and enhancing viability, whereas longer-term exposure with high doses reduced translocation and downregulated Bcl-2, leading to mitochondrial dysfunction and apoptosis [211].

Hydrocortisone enhanced mROS in healthy non-classical monocytes, but reduced the SA-induced rise in intermediate monocytes. Thus, glucocorticoids appear to have differential activation state and subtype-dependent effects; exposure of intermediate stimulated monocytes serves as a protective mechanism while it increases mROS generation in healthy non-classical cells.

Glucocorticoids may increase production of mROS both by enhancing metabolic rate and by decreasing the activity of antioxidant enzymes [543, 544]. Induction of mROS by glucocorticoids in lymphoid cells was observed as a later phenomenon than loss of MMP [545]. Disruption of MMP and generation of mROS is also involved in glucocorticoid-induced apoptosis in thymocytes, gingival tissue, and lymphoid cells, potentially caused by depletion or reduced expression of antioxidant enzymes [546-550].

In an *in vitro* model of mitochondria isolated from rat brain, dexamethasone but not hydrocortisone decreased superoxide generation [229]. An *in vitro* study in mouse macrophages found that both corticosterone and dexamethasone reduced LPS-

induced mROS by increasing UCP2 expression [535]. However, other studies have not confirmed that glucocorticoids do impact upon enzymatic or non-enzymatic antioxidants or oxidative damage [551-553].

3.4.2.3.2 Immune function

No effects on HLA-DR were found. Dexamethasone decreased HLA-DR expression at the transcriptional level. Pre-treatment with the GR inhibitor RU486 blocked these effects of dexamethasone. These discrepancies could potentially be explained by differences in duration, cell stimulation, and the type of glucocorticoid used. Dexamethasone has high affinity for the GR, whereas hydrocortisone has a lower affinity for the GR but also acts on MRs [554]. Similarly, it has been suggested that conflicting results on the use of glucocorticoids as an adjunctive therapy in sepsis could be explained by the type of glucocorticoid used, dose and duration of exposure, as well as activation state of the cells [490].

Although glucocorticoid effects are generally anti-inflammatory, their effects via genomic and non-genomic pathways differ by cell type [555]. Their effects on other aspects of the innate immune system are complex, but have been recently summarised in an excellent review [356]. In short, once bound to their receptor, the complex binds to GREs in the promoter regions of glucocorticoid-responsive genes. This results in transactivation, whereas binding to negative GREs results in suppression of target genes. Non-genomic effects include interaction with other transcription factors such as NF-kB and AP-1, or nuclear co-activators. Via these pathways, glucocorticoids generally down-regulate pro-inflammatory mediators, induce production of anti-inflammatory mediators, and differentiation of cells into anti-inflammatory phenotypes.

3.5 Strengths and limitations

With this *in vitro* model of infection, I aimed to study the effects of physiological levels of stress hormones, as seen in health and in septic patients, on immune cell mitochondrial and effector function. As with every *in vitro* model, this study comes with certain limitations, despite my best efforts to minimise these as much as possible.

Sepsis is a heterogenous syndrome, caused by a plethora of pathogens. My *in vitro* model only assessed hormone responses in PBMCs and monocytes, rather than investigating the interplay between organ systems, and I used only one type of bacteria (Gram-positive SA). The decision to use SA was however carefully made and based on initial optimisation studies with both Gram-positive and Gram-negative bacteria. Doses of HKB used in this and other *ex vivo* and *in vitro* studies far exceed what cells would encounter systemically, making direct translation to human sepsis difficult. The dose was however selected after initial dose titration studies.

Measurement of mitochondrial function, with analysis of MMP and mROS production, are relatively limited. Unfortunately, I had no time to measure O_2 consumption and glycolytic activity using respirometry, which may have added further information to the overall picture of immune cell mitochondria and metabolism in sepsis conditions. Similar comments apply to measurements of immune cell function. I only had time to analyse HLA-DR expression, as this is one of the most-studied markers of sepsis-induced immunosuppression. Additional analysis of phagocytosis, migration, bacterial clearance, and cytokine production and release would have added to the knowledge base of hormonal effects on immune cell function.

Also, I only looked at the effects of stress hormones after a 6-hour incubation. This timepoint was carefully selected based on initial optimisation studies, but could have diminished effects potentially seen with longer exposure times, such as genomic effects induced by glucocorticoids. In addition, I did not measure stress hormone levels in the supernatant after 6 hours' incubation as they could have potentially

degraded. However, these studies have been performed after a 24-hour incubation with catecholamines [193].

Lastly, there may well be type II errors due to the relatively low number of individual replicates. Some statistically significant differences have been missed simply due to insufficient numbers. However, I have noted dose-dependent changes in the preceding text. Another technical factor that could have diminished the effects of stress hormones on MMP in particular are the flow cytometer settings. I carefully set up these experiments with the Division of Medicine's flow cytometry expert. However, although TMRM emission in PBMCs was within range, subsequent analysis of monocyte subsets showed that the TMRM signal in intermediate monocytes moved to the far-right side of the spectrum.

Despite the above-mentioned limitations, I carefully considered the set-up of these experiments. The shift towards non-classical and intermediate monocytes after exposure to SA, and the distinct effects of stress hormones in the three sub-populations, highlights the importance of analysing these three subgroups separately, rather than looking at effects on total PBMCs and/or monocytes as a whole. Another strength of the current study is the use of multiple concentrations of hormones within the physiological range observed in septic patients. Although this might diminish effects that are normally seen with supraphysiological doses, for example by a recent publication by Stolk and colleagues (2020) [196], using realistic levels of exposure makes translation to the clinic more relevant.

3.5.1 Summary and conclusions

Gram-positive (SA) and Gram-negative (EC) HKB had differential effects on viability, with increased cell death after SA stimulation, though this may be related to higher concentrations of SA compared to EC. As incubation with SA caused more pronounced effects in MMP, mROS, and HLA-DR expression, I chose this bacterium

for further experiments looking at the modulating impact of different hormones on immune cell mitochondrial and effector function.

Differing dose-dependent effects were seen with hormone incubation in both unstimulated and SA-stimulated PBMCs and within monocyte subsets. These inconsistent effects varied according to cell type, activation state (with/without SA), and stress hormone concentrations. These differing responses may be related to the different specialised functions of these cells, the presence, affinity, and activation of α- and β-ARs and GRs on the cell types, and activation/suppression of downstream pathways.

I fully acknowledge the limitations of extrapolating such *in vitro* models to the clinical situation, nonetheless the doses of hormones were chosen to reflect physiological and pharmacological plasma levels rather than supra-pharmacological doses that are often used in other such studies [196]. In some cases, I found that the effects of HKB were augmented by stress hormones, e.g. a further decrease in MMP and augmentation of mROS production in some, but not all, monocyte populations. In other cases there was a reversal of effects, e.g. hydrocortisone increased mROS in healthy non-classical monocytes, but decreased mROS in stimulated intermediate monocytes. Different populations also responded differently, and dose dependency was also noted in some cases, with effects only seen with certain doses or in certain subtypes, but not with or in others.

These findings highlight the complexity of hormonal interactions with the immune system. In the clinical situation this will be compounded not only by changes in circulating levels of endogenous hormones and alterations in receptor/post-receptor sensitivity generated by the septic process, but also by iatrogenic contributions where hormonal treatments are often used in combination, e.g. noradrenaline and hydrocortisone in septic shock, and by other concurrent immunomodulating treatments such as sedatives and antibiotics. Whether these interventions are overall

beneficial or harmful in clinical practice is a matter of conjecture and likely fluctuate over time in an individual patient.

Catecholamines still need to be used therapeutically to restore blood pressure in shocked patients as no superior alternative has yet been found, and may therefore represent a necessary evil [556]. Corticosteroids, on the other hand, are not mandated treatments though are frequently used [8]. The clinical literature generally shows a reduction in vasopressor requirement with steroid use but variable impacts on overall mortality [461, 557-559]. Whether this relates to timing of intervention, dosing [489], or steroid type [493] is still uncertain. Furthermore, recent work in COVID-19 by the Calfee group shows different outcomes from corticosteroids, depending on the underlying inflammatory phenotype of individual patients [560].

The current study only analysed short-term effects of stress hormones, and included limited measurements of mitochondrial and immune function. In future experiments, I would like to include longer exposures and more markers of immune cell function, including cytokine production, phagocytosis, migration, and bacterial clearance. Respirometry could be employed as an additional measure of mitochondrial function and metabolism.

Chapter 4 Characterising endocrine, immune, and metabolic changes in a 3-day *in vivo* rat model of faecal peritonitis

4.1 Introduction

The endocrine, immune, and metabolic responses in an *in vivo* model of sepsis were assessed. These experiments were conducted before the current project started focusing on mitochondrial function of monocytes and did therefore not yet include specific markers of monocyte mitochondrial or effector function. The lab's well-established rat model of faecal peritonitis was re-characterised first to ensure previous results remained consistent in my hands. This included confirmation that an adequate dose of intraperitoneal (i.p.) faecal slurry injection had been administered, to ensure that a Stroke Volume (SV) threshold at 6-hours post-insult remained prognostic for survival. For determination of endocrine and inflammatory markers, blood was collected from septic (predicted survivors vs predicted non-survivors) and sham-operated control rats during the early (6 hours), established (24 hours), and recovery (72 hours) phases of sepsis. Animals were housed in individual metabolic cages for metabolic monitoring.

4.2 Methodology

4.2.1 Study design

A 3-day, fluid-resuscitated faecal peritonitis rat model of sepsis that was well-established within the lab was used. Personal (I213E6E67) and project licences (PPL 70/8290) granted by the UK Home Office were in place. Male Wistar rats (Charles River, Margate, Kent) weighing between 300-400 g were used for the experiments.

To reflect the clinical setting of sepsis in patients and improve translation of pre-clinical findings in our rat model, these experiments were in line with the 'Minimum Quality Threshold in Pre-Clinical Sepsis Studies' (MQTiPSS) proposed by Osuchokwski et al. [561]. Rats were acclimatised one week prior to instrumentation with *ad libitum* access to food and water. A 12-hour light/dark cycle was maintained during acclimatisation and further experiments. Rats were further acclimatised in individual metabolic cages 24 hours prior to anaesthesia and instrumentation.

During the initial survival study, to determine the dose of faecal slurry injection and the SV cut-off at 6 hours post-insult that enables prognostication of survival, blood was collected from sham-operated and surviving septic rats at 72 hours. Collection of blood from sham and septic rats (predicted survivors vs predicted non-survivors) at 6 and 24 hours was performed during a subsequent serum collection study with separate animals for each time-point. Animals were humanely culled after blood collection at the end of each experiment. An overview of the study design and procedures for both the initial study for prognostication of survival, and subsequent 6- and 24-hour serum collection studies, is illustrated in Figure 24.

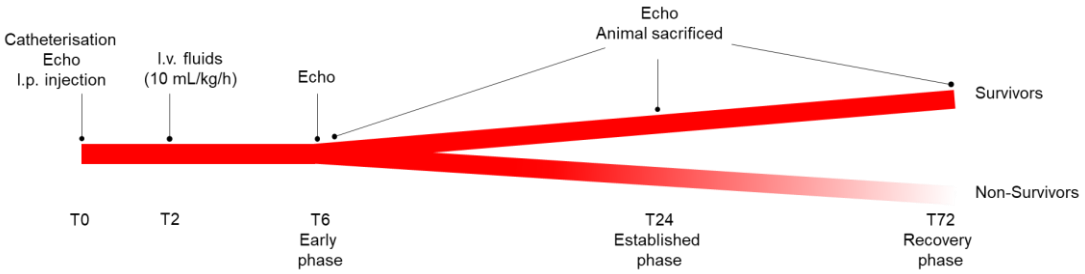

Figure 24. Study procedures during the *in vivo* rat model of faecal peritonitis. Animals were anaesthetised and underwent a baseline echo and catheterisation. Sepsis was induced by i.p. injection of faecal slurry. Sham operated control animals did not receive any injections. After 2 hours, i.v. fluids were initiated. At 6-, 24- and 72-hours animals underwent another echo and were culled for sample and tissue collection. Animals with sepsis were divided into survivors and non-survivors. T: time (hours); I.p.: intraperitoneal; I.v.: intravenous. Adapted from [417].

4.2.2 Instrumentation

Instrumentation and echocardiography were performed under isoflurane anaesthesia (Baxter Healthcare, Thetford, Norfolk) administered by a vaporiser (Vet-Tech Solutions, Congleton, Cheshire). Anaesthesia was induced by placing rats in an induction chamber spontaneously breathing 5% isoflurane. Maintenance was achieved using 2% isoflurane with rats spontaneously breathing through a nose cone. Adequate anaesthesia was confirmed by toe and tail pinch response. An additional subcutaneous (s.c.) injection of 0.025 mg/kg buprenorphine (Vetergesic, Reckitt Benckiser, York, Yorkshire) was given before and after instrumentation as long-acting analgesia. A rectal probe was inserted for continuous temperature monitoring during instrumentation and echocardiography. This probe was connected to a heated mat (TEST 1319, TES Electronical Corp, Taipei, Taiwan) to maintain a steady body temperature (36.5-37.5°C) during anaesthesia.

Hair was removed from the neck and chest using a razor and depilatory cream (Nair™, Church & Dwingt, Folkestone, Kent), and disinfected with 70% ethanol. A small incision of 2 mm length was made at the nape of the neck for tunnelling of the right internal jugular vein (Figure 25). This vessel was subsequently cannulated with 0.96 mm outer diameter Polyvinyl Chloride (PVC) tubing (Scientific Commodities, Lake Havasu City, AZ, USA) and secured within the vessel to a depth of 1-2 cm using 3-0 silk sutures (Ethicon, Bridgewater, NJ, USA). The other end was tunnelled s.c. to emerge at the small opening made at the nape of the neck. The skin incision sites were then sutured with single interrupted sutures (2-0 Ethilon, Ethicon) and cleaned with 70% ethanol.

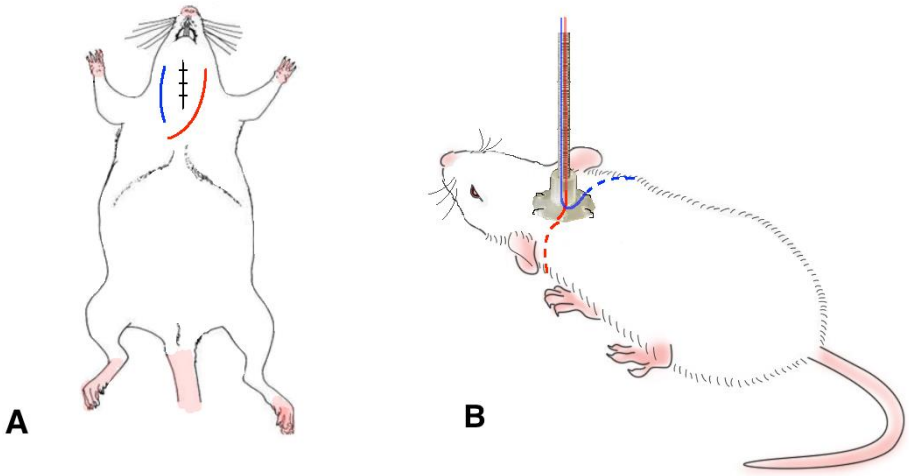

Figure 25. Venous and arterial lines inserted through midline neck incision and tunnelled subcutaneously to the neck, exiting through an external tether. In current experiments, only venous lines were used. Adapted from [417].

The tubing was flushed with heparinised (2000 IU/L) (Fannin) 0.9% saline (Baxter Healthcare) before use to prevent clotting, and connected to a swivel tether system (InsTech Solomon, Plymouth Meeting, PA, USA) (Figure 26). The tether system was secured to the animal with a silicone jacket whilst still anaesthetised. After instrumentation and i.p. injection, the swivel-tether system was attached to a balancing arm on top of the individual metabolic cages to allow free movement of animals within their cage.

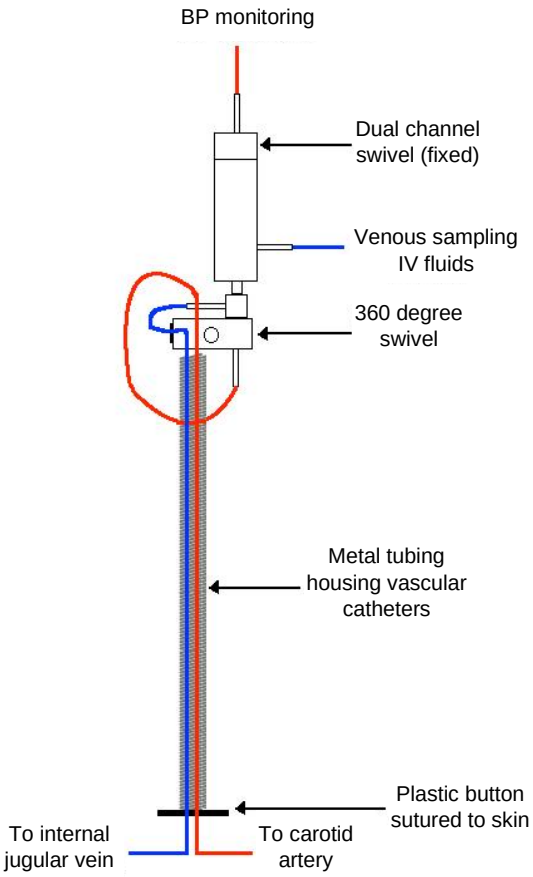

Figure 26. Swivel-tether system for arterial and venous lines, allowing free movement. In current experiments, only venous lines were used. BP: Blood Pressure; IV: Intravenous. Adapted from [417].

4.2.3 Sepsis induction by faecal peritonitis

Sepsis was induced by i.p. injection of faecal slurry. The slurry was obtained from pooled and processed stool samples from four healthy human volunteers. Faecal slurry preparation was executed using a protocol developed by Bauer and Claus at Jena University Hospital, Jena, Germany. Microbiological analysis of aliquots of the current batch prepared in 2018 was kindly performed by the UCLH Clinical Microbiology Laboratory, and compared to our lab's old batch from 2013.

During slurry preparation, stool samples were kept on ice and continuously fumigated with nitrogen. The total amount of stool was weighed, and a broth of thioglycollate medium of 29.8 g/L (Merck Millipore, Darmstadt, Germany) in 0.9% NaCl (Sigma-

Aldrich) was added in the same amount as the total stool weight. Barium sulphate (Thermo Fisher Scientific) and glycerol (Sigma-Aldrich) were subsequently added as 10% of total weight, and 165 uL of catalase from bovine liver (Sigma-Aldrich). Another 100 mL of 0.9% saline (Sigma-Aldrich) was added, and the stool mixture was then homogenised, aliquoted into 50 mL Falcon tubes, and stored at -80°C. One aliquot of faecal slurry was divided on ice into smaller 1 mL aliquots in pre-cooled Eppendorf tubes under continuous fumigation with nitrogen and stored at -80°C.

These small batches of faecal slurry enable an identical septic insult to be given to each animal. Sepsis was induced by i.p. injection of faecal slurry using a 19-gauge needle into the right lower quadrant of the abdomen. Faecal slurry was first diluted 1:4 with 0.9% normal saline (Baxter Healthcare), and warmed to 37°C before injection. An initial pilot study by colleagues in the lab determined the appropriate dose of slurry, according to the weight of the animal, to produce a 72-hour mortality of approximately 40%, without animals dying within the first 24 hours. This reflects an approximate mortality rate of septic shock from faecal peritonitis in humans [562]. Successful injection was visually confirmed at the end of each study. Sham-operated control animals received no i.p. injection to avoid accidental bowel perforation.

4.2.4 Fluid resuscitation

Animals received intravenous (i.v.) fluids after surgery over the entire duration of the study. The venous line was connected to an infusion pump and flushed continuously to maintain potency with 0.1 mL/h of heparinised (2000 IU/L) (Fannin) 0.9% saline (Baxter Healthcare) for the first 2 hours. Two hours following surgery, a continuous fluid resuscitation was initiated at a rate of 10 mL/kg/h with a 1:1 ratio of 5% glucose (Baxter Healthcare) and Hartmann's solution (Baxter Healthcare) to avoid hypoglycaemia. At 6 hours, a fluid bolus of 20 mL/kg was administered over 2

minutes, and another fluid bolus of 10 mL/kg over 1 minute at 24 hours. Fluids were subsequently reduced by half every 24 hours until the end of experiment.

4.2.5 Metabolic cage monitoring

Prior to surgery and instrumentation, rats were acclimatised in individual metabolic cages for 24 hours (Figure 27). After instrumentation, animals were allowed to awaken again in these same cages. The cages were connected to a Comprehensive Lab Animal Monitoring System (CLAMS Oxymax) (Columbus Instruments, Columbus, OH, USA) which supplied fresh room air via flow controllers and an air inlet, and monitored O_2 consumption and CO_2 production at the outlet sampling lines of each cage. Gas samples were dried, enabling the system to detect O_2 through a gas sensor that generates small amounts of electricity when in contact with O_2. The electrical signal was subsequently sensed by the circuit built into the CLAMS system. CO_2 was sensed by a single-beam non-dispersive infrared light within the system.

This system was stabilised for at least 3 hours prior to onset of each experiment. Sensors were calibrated using a gas mix of 20.5% O_2 and 0.5% CO_2. Whole-body O_2 consumption (VO_2) and CO_2 production (VCO_2) were calculated by the system as the difference between inlet and outlet flows. These values were normalised against each rat's individual body weight. Subsequently, the Respiratory Exchange Ratio (RER) could be calculated as the ratio of volume of CO_2 produced to volume of O_2 used.

Figure 27. Metabolic cage monitoring setup with CLAMS Oxymax equipment, individual metabolic cages, and infusion pumps. Adapted from [417].

4.2.6 Clinical scoring

To prevent unnecessary animal suffering and breaching study protocol severity, animals were monitored at least 4 times a day during the experiment. A scoring system had been developed in conjunction with UCL's BSU and approved by the UCL Veterinary Surgeon and Home Office Inspector. This system was used to assess various characteristics, including appearance, behaviour, clinical signs, and other observations (Supplementary Table 2). In case animals scored 2 points for any of the criteria listed, monitoring was increased to once every 2 hours. If no clinical improvement was observed within the next 8 hours, the experiment was terminated, and animals culled to prevent further suffering. Animals scoring 3 or higher for any of the criteria were culled immediately.

4.2.7 Echocardiography

Previous experiments in our lab, and repeated by several investigators, have shown that echocardiography-derived SV, as soon as 3-6 hours after sepsis induction, is an accurate predictor of 72-hour mortality [563]. All animals in the current study underwent echocardiography at baseline, 6 hours post-sepsis induction (to

prognosticate), and for longer experiments at the end of the experiment at 24 or 72 hours. Animals were induced with 5% isoflurane as described earlier. Anaesthesia during echocardiography was maintained using 1.5% isoflurane, while rats were placed on a heating mat with continuous monitoring of core temperature.

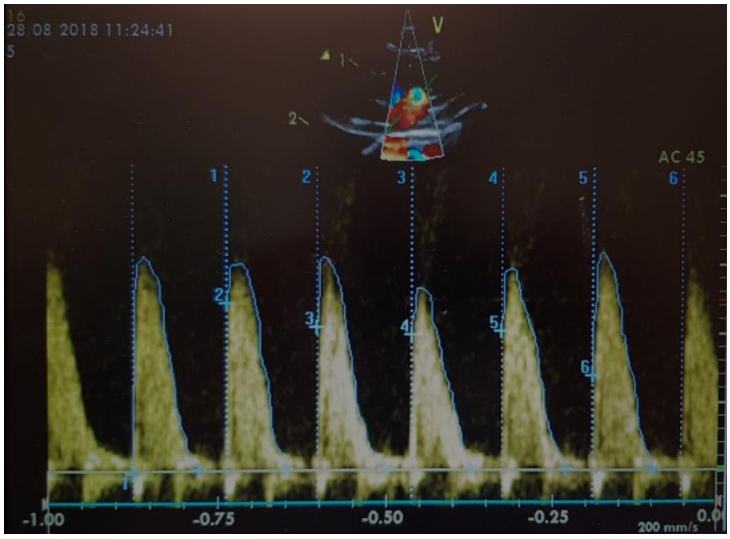

Figure 28. Pulsed wave Doppler measurement of aortic blood flow. The waveform displays blood flow velocity against time (Velocity-Time Integral (VTI)). The area under each waveform is proportional to SV.

Transthoracic Echocardiography (TTE) was performed using a Vivid 7 Dimension device (GE Healthcare) and a 10 MHz sector transducer (Vivid, 10S, GE Healthcare). Aortic blood flow was measured by pulsed wave Doppler at the point of bifurcation of the right carotid artery from the aortic arch (Figure 28). Blood flow direction was confirmed using colour Doppler. The area under each waveform obtained by pulsed wave Doppler is the Velocity-Time Integral (VTI). Due to variability of HR with respiration, an average VTI was taken after measuring the area under 6 consecutive waveforms.

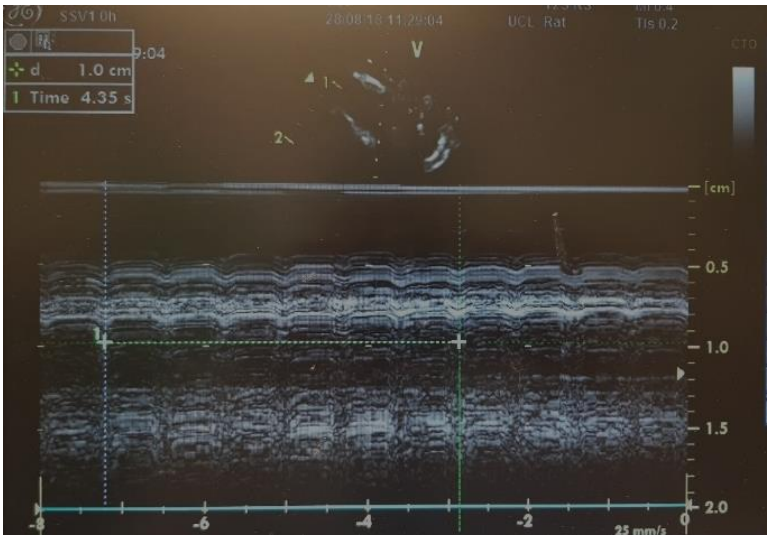

Figure 29. M-mode trace of diaphragm movement to measure respiratory rate. Six consecutive cycles were measured and averaged to calculate the respiratory rate.

SV was calculated using the equation SV = 0.25 π d^2 (VTI), where *d* is aortic diameter. The aortic diameter was previously established using rats of the same age and weight. A mean diameter of 0.26 cm was subsequently used for calculating SV in the current study. HR could also be derived from these measurements by measuring the time between the start of each Doppler waveform over six consecutive cycles. HR and SV could subsequently be used to calculate CO using the formula CO = SV x HR. M-mode ultrasound of the diaphragm was used to determine the RR, using at least six consecutive respiratory cycles (Figure 29).

4.2.8 Sample collection

Blood samples from septic and sham-operated control animals were collected at the end of the experiment for both the initial 72-hour characterisation of the model, and the subsequent 6- and 24-hour experiments. Rats were culled at one of the three pre-determined time-points (6, 24 or 72 hours), or when their clinical severity scores necessitated an early cull. These timepoints were chosen to reflect the early, established, and recovery phases of sepsis, respectively.

Rats were anaesthetised as described previously. A rectal probe was inserted for continuous temperature monitoring. This probe was connected to the heated mat for maintenance of body temperature during anaesthesia. Sutures on their chest wall were removed and blood was collected via surgical cannulation of the left carotid artery prior to culling. In case of impossible arterial blood withdrawal, heart puncture was performed. Blood was collected into sterile 15 mL Falcon tubes for serum separation. After 30 minutes of clotting at room temperature, tubes were centrifuged at 4000 RPM for 15 minutes. Serum was separated and pipetted into clean Eppendorf tubes, snap frozen in liquid nitrogen, and stored at -80°C. In addition, 0.2 mL of blood was collected into heparin-coated capillary tubes for Blood Gas Analysis (BGA) (ABL-700, Radiometer, Copenhagen, Denmark).

4.2.9 Biochemical analyses

Multiple biochemical analyses were performed to assess the endocrine and immune response during sepsis in rat serum samples, using commercially available ELISA kits. To optimise accuracy of these measurements, four samples of serum from both sham and septic rats were initially tested in three different dilutions. The sample dilution providing the most accurate detection of the analyte was subsequently used to perform all further analyses for that particular analyte. All ELISAs were performed according to the relevant instruction manuals.

Signal quantification of the 96-well hormone ELISAs were measured spectrophotometrically using a Synergy 2 plate reader (Biotek) set to the appropriate wavelength. Origin 2019 software (OriginLab Corp.) was used to produce a four-parameter logistic curve from the OD values of the reference standards for the hormone ELISAs, allowing determination of sample analyte concentrations.

Competitive ELISA kits were used to assess levels of adrenaline, noradrenaline, glucagon, fT_3, and rT_3 (all Elabscience Biotechnology Co.). For measurement of

adrenaline (E-EL-0045), noradrenaline (E-EL-0047), and glucagon (E-EL-R0425), a 1:5 dilution of serum in sample diluent was used. For determination of fT_3 (E-EL-0079) and rT_3 (E-EL-R1420), dilutions of 1:50 and 1:25 were used, respectively. All these readings were performed at a wavelength of 450 nm.

Analysis of insulin and corticosterone levels were performed using ELISA kits from different suppliers. Insulin levels were analysed in a 1:6 dilution of serum in sample diluent, using a sandwich ELISA kit (EZRMI-13K, EMD Millipore, Watford, Hertfordshire) and read at 450nm. For measurement of corticosterone levels, samples were also diluted 1:6 and a competitive ELISA kit was used (ADI-900-097, Enzo Life Sciences, Exeter, Devon). Readings for this ELISA kit were performed at 405 nm.

In addition, rat specific solid-phase sandwich DuoSet™ ELISAs were used for measurement of predominantly pro-inflammatory cytokine IL-6, and the predominantly anti-inflammatory cytokine IL-10 (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cytokine standards were reconstituted in Reagent Diluent (1% BSA in PBS) with 20% FBS, to correct for any serum matrix effects. Serum samples were diluted 1:5 and plates read at both 450nm and 540nm for wavelength correction using the lab's SPECTROstar spectrophotometer (BMG Labtech Ltd., Aylesbury, Buckinghamshire). Cytokine concentrations were subsequently calculated based on interpolation of a four-parameter logistic curve using Prism Version 9 (GraphPad Software).

4.2.10 Statistical analysis

Data were organised and summarised using Office Excel (Microsoft). GraphPad Prism Version 9 (GraphPad Software) was used for statistical analysis and data visualisation. Normality was assessed by inspection of QQ plots and Shapiro-Wilk test for normality, with parametric or non-parametric tests used accordingly. When comparing three groups at one time-point, one-way ANOVA with Tukey's multiple

comparisons correction or the non-parametric Kruskal-Wallis test with Dunn's multiple comparisons correction were used. When comparing two groups at one time-point, Student's t-test or the non-parametric Mann-Whitney test were used. Analysis of respirometry data was performed with proprietary Wave software (Agilent, Santa Clara, CA, USA). A *P*-value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 *In vivo* model characterisation

4.3.1.1 Faecal slurry preparation and dose titration

A new batch of faecal slurry was prepared in 2018 for i.p. injection and induction of faecal peritonitis as a model of sepsis. Microbiological analysis was performed by the UCLH Clinical Microbiology Lab on aliquots of faecal slurry prepared in 2018, and compared to analysis of faecal slurry from 2013.

The analysis shows a similar order of bacteria for both batches, but a slightly higher Colony Forming Unit (CFU)/mL for the batch prepared in 2018. Differences were also found in the types of aerobic and anaerobic bacteria present (Supplementary Table 3). Prior microbiological studies in the lab have confirmed long-term viability and reproducibility of faecal flora after different periods of storage at -80°C (data not shown).

Characterisation of the *in vivo* model of faecal peritonitis with the new batch of faecal slurry was performed by colleagues in the lab. The aim was to determine the right dose of faecal slurry to generate a mortality rate of approximately 40% at 72 hours, similar to mortality rates of sepsis in humans [562]. Increasing doses of slurry were used, ranging from 0 to 9 mL/kg body weight. An increasing dose of faecal slurry produced a progressive increase in mortality, with a dose of 7 mL/kg body weight producing a mortality rate of 55% (*n*=11) (Table 5). This dose was subsequently

chosen for my experiments, producing a similar 72-hour mortality rate of 56% (*n*=16) (Figure 30).

Table 5. 72-hour mortality with different doses of i.p. faecal slurry. Increasing doses of faecal slurry were used by colleagues in the lab to generate a mortality rate similar to that in human sepsis. A dose of 7 mL/kg was subsequently chosen.

Dose (mL/kg)	**Rats (*n*)**	**72-hour mortality**
0 (sham)	6	0%
4	3	0%
6	6	16%
7	11	55%
9	6	67%

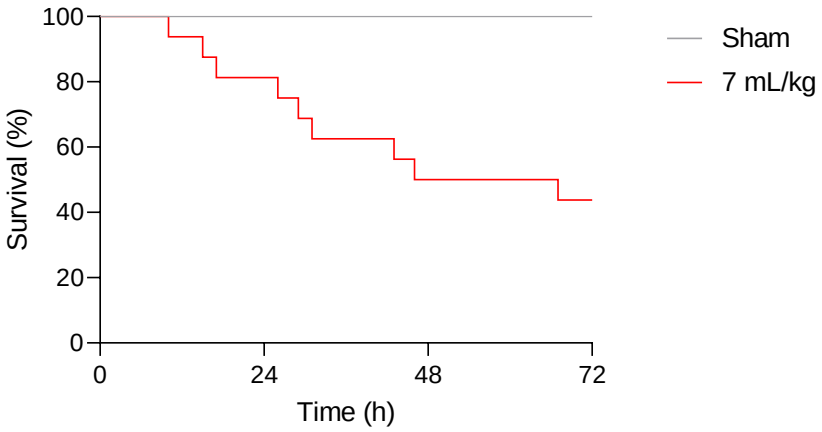

Figure 30. Kaplan-Meier 72-hour survival curve (Log Rank *P*=0.03). A faecal slurry dose of 7 mL/kg produced a 72-hour mortality rate of 55% compared to 0% mortality in the sham operated control animals.

4.3.1.2 Validation of echocardiography

During validation experiments, Doppler echocardiography was performed at baseline, during early sepsis (6 hours), and at the end of the experiment (72 hours) for sepsis survivors and sham-operated control animals. Based on these echocardiographic measurements, SV, HR, and CO could be determined (Table 6).

Table 6. Echocardiography of survival study. Echocardiography was performed at baseline (0h), and 6 and 72 hours after catheterisation in rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. CO was calculated from HR and SV. Data presented as mean ± SD. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor; h: hour. ****P<0.0001 vs control; ^{+}P<0.05 vs SS.

		Sham (n=6)	**SS (n=7)**	**SNS (n=9)**	***P*-value**
HR (beats/min) NR: 300-380	**0h**	417 ± 30	452 ± 26	455 ± 33	0.06
	6h	450 ± 22	465 ± 31	463 ± 37	0.66
	72h	446 ± 31	421 ± 30		0.18
SV (mL) NR: >0.35	**0h**	0.45 ± 0.06	0.40 ± 0.07	0.49 ± 0.05^{+}	0.03
	6h	0.44 ± 0.04	0.27 ± 0.05****	0.21 ± 0.03****$^{+}$	<0.0001
	72h	0.43 ± 0.02	0.43 ± 0.07		0.84
CO (mL/min) NR: >130	**0h**	187 ± 15.11	178 ± 29	220 ± 23^{+}	0.02
	6h	198 ± 11.42	123 ± 23****	98 ± 10 ****$^{+}$	<0.0001
	72h	194 ± 10.31	181 ± 34		0.38

Measurements of SV and HR at baseline and 6 hours after sepsis induction and instrumentation have previously shown to be reliable markers for prognostication of survival [563, 564]. However, in my current model, no changes in HR at 6 hours were observed between sham-operated controls and septic animals (P=0.66). HR measurements at baseline were higher in both predicted survivors and non-survivors, but the difference against sham control animals did not reach significance (P=0.12 and P=0.06, respectively). HR in all groups however exceeded the normal range for rats of similar age and weight (300-380 beats/min).

A significant decrease in SV was found in both groups of septic rats when compared to sham-operated controls at 6 hours (P<0.0001 for both), with a greater decrease in those animals who go on to die (P=0.03). At 6 hours, SV fell below the normal range (>0.35 mL) for both septic groups, but recovered at 72 hours in the surviving animals. CO followed a similar trend, with significantly higher levels in predicted non-survivors when compared to survivors even at baseline (P=0.04). However, at 6 hours, CO in

predicted non-survivors dropped markedly compared to predicted survivors (*P*=0.01). Values in both predicted survivors and non-survivors also differed significantly from sham-operated control animals (*P*<0.0001 for both).

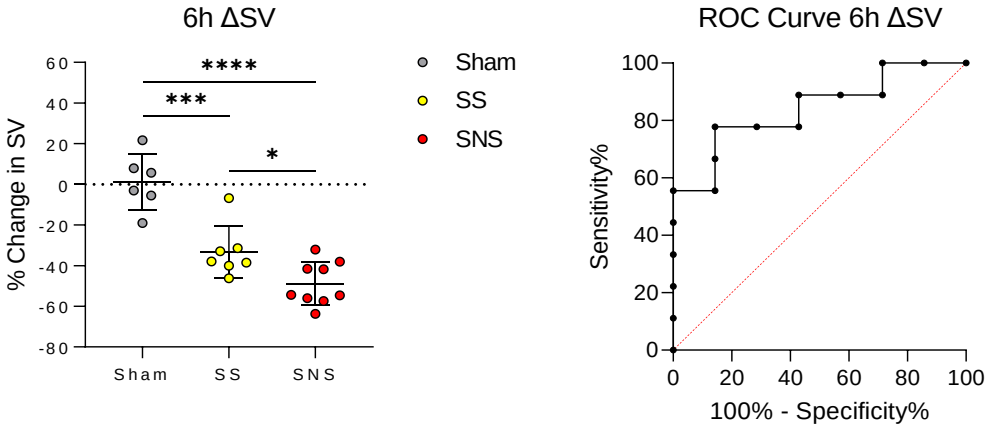

Figure 31. Difference in SV as prognosticator for survival at 72 hours. Echocardiography was performed at baseline (0h) and 6 hours after catheterisation in rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. The %change in SV was calculated by subtracting SV at 6 hours from SV at baseline, and subsequently dividing this number by SV at baseline. Data presented as mean ± SD. SV: Stroke Volume; ROC: Receiver Operating Characteristic; SS: Sepsis Survivor; SNS: Sepsis Non-Survivor; h: hour. **P*<0.05; ****P*<0.001; *****P*<0.0001.

As SV already differed between predicted non-survivors and survivors at baseline (*P*=0.02), the percentage change in SV from baseline to 6 hours was used as a predictor of mortality (Figure 31). This measure had an Area Under the ROC (AUROC) curve of 0.84 (Confidence Interval (CI) 0.61-1). A cut-off value of a 40.8% fall in SV from baseline was associated with a sensitivity of 78% (CI 45-96%) and specificity of 86% (CI 49-99%). This cut-off was subsequently used to assign predicted mortality to septic animals in further experiments.

4.3.1.3 Metabolic cage monitoring

After instrumentation, animals were individually housed in metabolic cages, enabling measurement of VO_2, VCO_2 and subsequent calculation of the RER. A clear diurnal pattern can be seen in RER values in sham-operated controls, with higher RER values overnight compared to the day, which corresponds to periods of increased activity (Figure 32). RER values in sham animals ranged from 0.9-1.0, indicating predominant use of carbohydrate metabolism. A very early drop in RER and loss of diurnal rhythm was seen in both septic groups. Even in septic survivors, the diurnal rhythm was not restored by study end (72 hours). RER values for septic animals ranged from 0.75-0.85, indicating a shift towards utilisation of protein (RER 0.83) and fat (RER 0.70).

VO_2 levels were lower in septic animals compared to sham operated controls, but were initially similar in survivors and non-survivors. Divergence commenced approximately 24 hours after the induction of sepsis. At this timepoint, the VO_2 of surviving animals returned to normal, while that of non-survivors continued to decrease further. A VO_2 reading of <900 mL/kg/h was associated with mortality in all animals. The VCO_2 profile was comparable to the VO_2 profile in septic animals, with the only difference being that VCO_2 values in septic survivors did not normalise by study end (72 hours) (Figure 32).

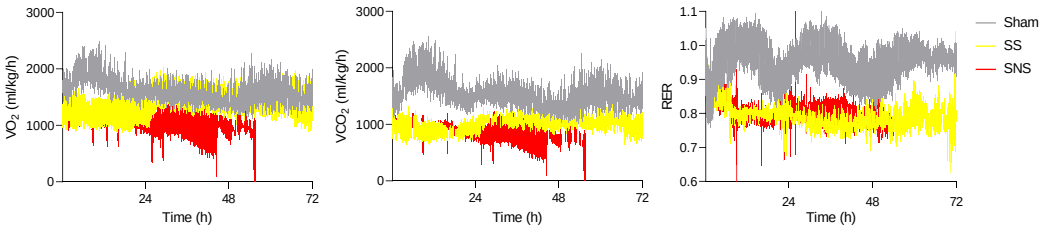

Figure 32. VO_2 consumption, VCO_2 production, and RER during survival study. Animals were continuously monitored in metabolic cages after catheterisation. These graphs depict rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. RER was calculated from VO_2 and VCO_2. Data presented as mean ± SD. VO_2: O_2 Consumption; VCO_2: O_2 Production. RER: Respiratory Exchange Ratio; SS: Septic Survivor; SNS: Septic Non-Survivor; h: hour. Sham *n*=6; SS *n*=7; SNS *n*=9.

4.3.2 *In vivo* serum collection study

4.3.2.1 End-physiological and haemodynamic parameters

For all animals, in addition to measurements at 0 and 6 hours to prognosticate survival, echocardiography was also performed at the end of each experiment (at either 6, 24 or 72 hours) prior to blood collection. To be able to interpret a more overall picture of haemodynamics, measurement of Hb, O_2 Saturation (sO_2), and the pO_2 by BGA were also included in Table 7.

No significant differences were observed in HR between the groups at any timepoint. Rates however exceeded the normal range for all groups at all timepoints (300-380 beats/min). Significant differences were found in SV at 6 hours between sham-operated control animals, predicted survivors (P<0.0001) and non-survivors (P<0.0001), and also between the two septic groups (P=0.01). SV for both septic groups fell below the normal range (>0.35 mL), but recovered in predicted survivors at 24 and 72 hours. CO at 6 hours, as derived from HR and SV, was also significantly higher in sham-operated controls compared to predicted non-survivors (P=0.0002). Levels in both septic groups fell below the normal range at 6 hours (>130 mL/min).

A significant decrease in RR was found at 6 hours in predicted non-survivors compared to sham operated controls (P=0.01), but recovered at later timepoints. RR in sham controls, predicted septic survivors and non-survivors at 6 and 72 hours all exceeded the normal range (45-65 breaths/min). A trend towards decreased temperatures in septic animals was seen at 6 and 24 hours, but this did not reach significance (P=0.07 for both time-points).

The Hb of arterial and mixed blood obtained via heart puncture was significantly higher in both septic groups at 6 hours compared to sham-operated control animals (P=0.0007 for predicted survivors, P=0.004 for non-survivors), despite significant fluid loading from 2 hours, but decreased at subsequent timepoints.

Table 7. End physiological and haemodynamic parameters. Echocardiography was performed and whole blood taken at 6, 24 and 72 hours after catheterisation in rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham controls to measure HR, SV, and RR. CO was calculated from HR and SV. Temperature was measured using a rectal probe. Hb, sO_2 and pO_2 were measured by BGA of whole blood. DO_2 was subsequently calculated. NR: Normal Range; SS: Sepsis Survivor; SNS: Sepsis Non-Survivor; h: hour. **P*<0.05; ***P*<0.01; ****P*<0.001; *P*<0.0001 vs sham. $^{++}$*P*<0.01 vs SS.

		Sham	**SS**	**SNS**	***P*-value**
HR (beats/min) NR: 300-380	**6h**	440 ± 20	430 ± 42	462 ± 38	0.31
	24h	423 ± 53	480 ± 55	455 ± 51	0.26
	72h	446 ± 31	421 ± 30		0.18
SV (mL) NR: >0.35	**6h**	0.45 ± 0.06	0.29 ± 0.04****	0.20 ± 0.02******$^{++}$	<0.0001
	24h	0.35 ± 0.06	0.36 ± 0.05	0.33 ± 0.07	0.65
	72h	0.44 ± 0.02	0.43 ± 0.07		0.84
CO (mL/min) NR: >130	**6h**	196 ± 24	124 ± 16	94 ± 12***	<0.0001
	24h	147 ± 25	171 ± 8	149 ± 40	0.46
	72h	194 ± 10	181 ± 34		0.38
RR (breaths/min) NR: 45-65	**6h**	71 ± 16	61 ± 8	49 ± 6*	0.02
	24h	76 ± 20	75 ± 17	70 ± 13	0.76
	72h	90 ± 9	76 ± 14		0.14
Temperature (°C) NR: 36.1-37.3	**6h**	37.9 ± 0.4	37.2 ± 0.6	37.5 ± 0.4	0.07
	24h	37.8 ± 0.4	36.4 ± 2.5	35.5 ± 1.7	0.07
	72h	37.8 ± 0.5	37.5 ± 0.3		0.42
Hb (g/dL) NR: 13.5-16.0	**6h**	12.7 ± 0.7	16 ± 0.9***	15.7 ± 2.0**	0.0005
	24h	13.1 ± 0.6	14 ± 1.1	14.2 ± 1.5	0.28
	72h	12.2 ± 0.4	12 ± 0.8		0.35
sO_2 (%) NR: >91%	**6h**	85.2 ± 14.7	82.4 ± 13.1	92.1 ± 5.0	0.51
	24h	90.2 ± 3.7	54.0 ± 38.6	53.2 ± 34.7	0.07
	72h	91.4 ± 2.4	93.1 ± 2.5		0.30
pO_2 (kPa) NR: 10.2-12.4	**6h**	10.8 ± 4.4	12.7 ± 4.8	10.8 ± 2.2	0.63
	24h	11.8 ± 3.2	10.9 ± 8.9	7.1 ± 3.6	0.20
	72h	11.0 ± 1.0	12.4 ± 1.7		0.13
DO_2 (mL/min) NR: >22	**6h**	29.0 ± 8.2	21.2 ± 3.2	18.14 ± 3.0*	0.01
	24h	23.4 ± 4.6	18.6 ± 12.6	16.3 ± 8.6	0.35
	72h	29.1 ± 2.4	26.2 ± 6.4		0.32

Initially, sO_2 was similar in all groups at 6 hours, with slightly low saturation in sham control animals and predicted survivors compared to the normal range (>91%). Where saturation increased to normal range in sham animals, it seemed to decrease at 24 hours in both septic groups. However, this did not reach significance (*P*=0.07). At 72 hours, saturation in predicted survivors was restored.

The calculated total amount of O_2 Delivered (DO_2) to tissues per minute was significantly lower at 6 hours in predicted non-survivors compared to control animals (*P*=0.01). The fall in predicted survivors did not reach significance (*P*=0.07). Levels at 24 hours remained lower in septic animals compared to the normal range, but returned back to normal at 72 hours in predicted survivors (>22 mL/min). It must be noted that a heartpuncture had to be performed for *n*=2 septic animals at 6 hours, *n*=6 septic animals at 24 hours, and *n*=1 septic animal at 72 hours. This means results cannot always be directly compared between groups and with normal ranges for arterial blood.

4.3.2.2 Blood gas analysis

BGA was performed on arterial whole blood and occasionally mixed blood derived from heartpuncture at the end of each experiment at the three pre-determined time-points. Glucose levels were initially similar for all three groups at 6 hours, but decreased at 24 hours for sepsis survivors (Kruskal Wallis: *P*=0.001; Dunn's: *P*=0.02) and non-survivors (*P*=0.01), without significant differences between the two septic groups. The significant difference between sepsis survivors and sham-operated control animals persisted at 72 hours (*P*<0.0001).

Similarly to glucose levels, lactate levels were initially similar in all groups. However, at 24 hours, compared to sham-operated controls, lactate levels were significantly higher in sepsis survivors (ANOVA: *P*=0.03; Tukey's: *P*=0.05) and non-survivors

(*P*=0.04), without any differences between the latter two groups. At 72 hours, lactate levels remained higher in sepsis survivors compared to sham animals (*P*=0.02).

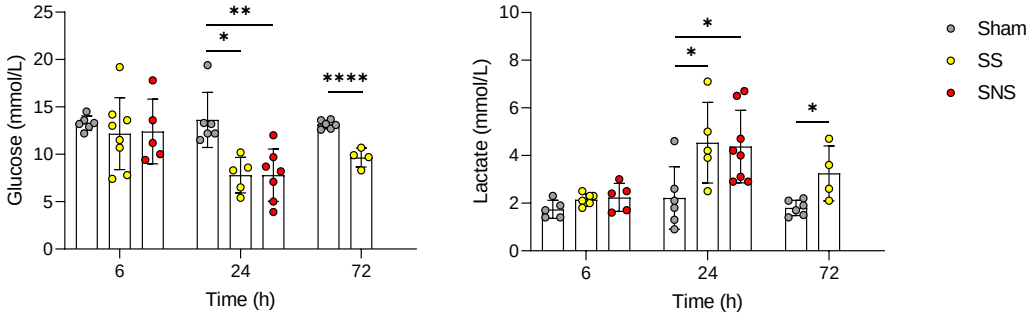

Figure 33. Blood levels of glucose and lactate. Glucose and lactate were measured by BGA of whole blood taken at 6, 24 and 72 hours after catheterisation from rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. Levels are presented as mean ± SD. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor; h: hour. **P*<0.05; ***P*<0.01; *****P*<0.0001.

Compared to sham controls, pH fell at 24 hours in predicted non-survivors (*P*=0.02) (). This was related to a metabolic acidosis with an increase in base deficit, fall in HCO_3^-, and rise in lactate (Figure 33). The maximal effect on base deficit was seen at 6 hours but improved to some degree at 24 hours, likely related to the ongoing fluid resuscitation which commenced at 2 hours. Although pCO_2 levels seemed to rise in the septic animals, this did not reach statistical significance. Of note, the septic non-survivors showed decreasing pO_2 and a rising pCO_2 at 24 hours, suggestive of worsening respiratory failure which may in part be related to increasing fatigue and decreasing effort.

Electrolyte levels were deranged at 6 hours in the septic animals with lower Na^+ and higher K^+ values, but they had corrected by 24 hours. No clinically relevant changes were seen in Ca^{2+}, Cl^-, or HCO_3^-. The bilirubin levels were supranormal in all groups, however there was no difference between sham and septic animals. This may possibly be due to a mechanical haemolysis related to blood extraction through the

arterial catheter, which may also explain the rise in potassium. However, the potassium levels were within the normal range rather than supranormal.

4.3.2.3 Hormone measurements

Levels of various stress and metabolic hormones were measured by ELISA in serum collected from septic (predicted survivors vs non-survivors) and sham-operated controls rats at three pre-determined time-points, representing the early (6 hours), adaptive (24 hours), and recovery (72 hours) phases of sepsis. Of note, the hormone levels were significantly above the assay manufacturer's reference ranges, despite excellent standard curves being obtained. Whether these given ranges are inaccurate or not applicable to this particular species is uncertain, but differences between sham controls and septic predicted survivors and non-survivors are likely more relevant.

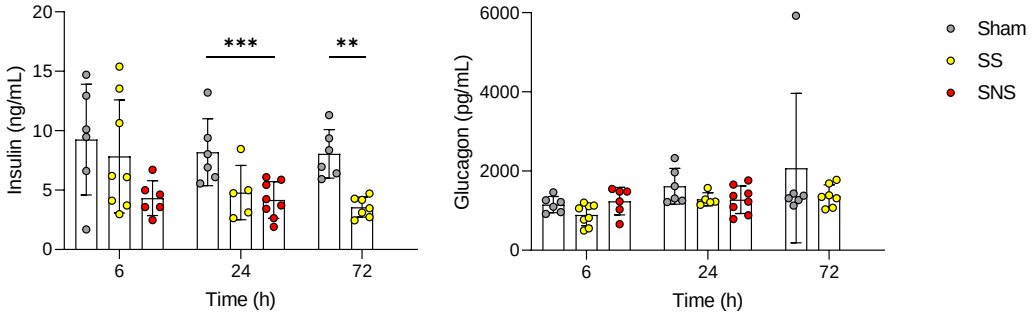

Figure 34. Serum levels of insulin and glucagon. Hormones were measured by ELISA in serum samples taken at 6, 24 and 72 hours after catheterisation from rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. Data presented as mean ± SD. SS: Septic Survivor; SNS: Septic Non-Survivor; h: hour. ***P*<0.01; ****P*<0.001.

Insulin levels were significantly lower in predicted non-survivors compared to sham-operated control animals at 24 hours (ANOVA: *P*=0.01; Tukey's: *P*=0.01), and in predicted survivors at 72 hours (*P*=0.0002). However, insulin levels in all groups exceeded normal reference values (1.4-3.7 ng/mL) for healthy rats of similar age and weight. No significant differences in glucagon levels were observed between sham-

operated control rats and septic animals. Glucagon levels at all time-points exceeded normal reference values of 66-98 pg/mL (Figure 34).

Normal reference values for noradrenaline and adrenaline range from 0.2-0.6 ng/mL and 0.1-0.9 ng/mL, respectively. Levels in both control and septic animals far exceeded these reference values in both groups. Despite no evidence of clinical distress in the sham animals, this may indicate in part a psychological stress response related to being tethered and isolated from the other rats, which did not settle over time for noradrenaline, but did for adrenaline (Figure 35).

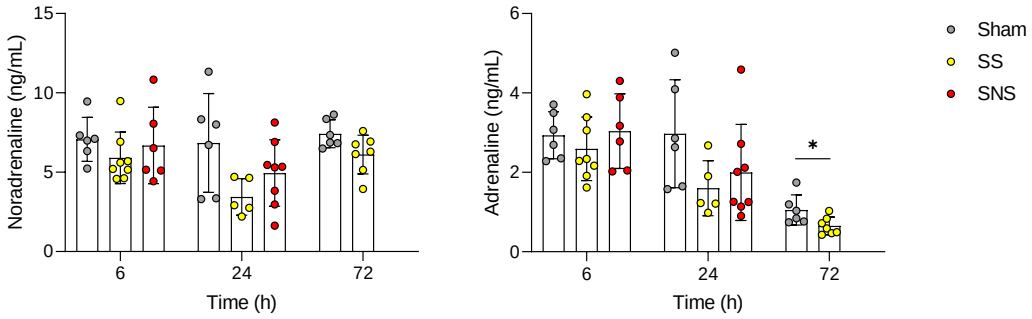

Figure 35. Serum levels of noradrenaline and adrenaline. Catecholamines were measured by ELISA in serum samples taken at 6, 24 and 72 hours after catheterisation from rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. Data presented as mean ± SD. SS: Septic Survivor; SNS: Septic Non-Survivor; h: hour. *P<0.05.

Levels of fT_3 in both control and septic rats exceeded reference values of 3.2-4.8 pg/mL at all timepoints. Significant differences between groups were seen at 24 hours, with an increase in fT_3 and a decrease in rT_3 (Figure 36). Levels of fT_3 were higher in predicted non-survivors compared to control animals (ANOVA: P=0.002; Tukey's: P=0.001), whereas levels of rT_3 were higher in sham operated animals compared to both predicted survivors (ANOVA: P=0.003; Tukey's: P=0.008) and non-survivors (P=0.004).

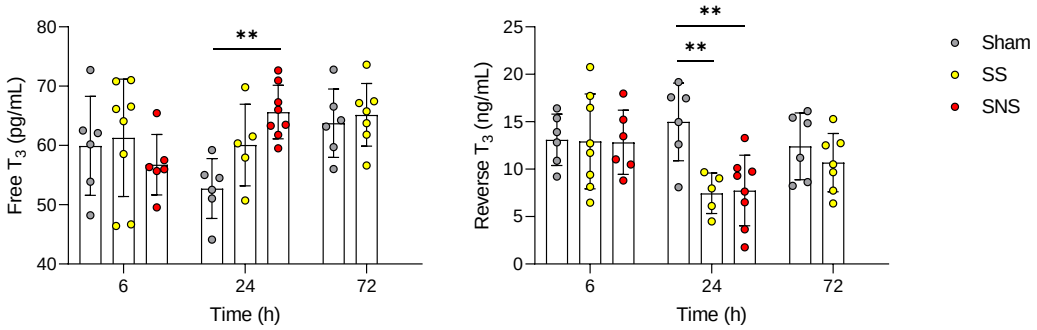

Figure 36. Serum levels of fT_3 and rT_3. Thyroid hormones were measured by ELISA in serum samples taken at 6, 24 and 72 hours after catheterisation from rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. Data presented as mean ± SD. SS: Septic Survivor; SNS: Septic Non-Survivor; h: hour. ***P*<0.01.

The reference range for early morning corticosterone is reported as 31-114 ng/mL. Corticosterone levels increased in septic animals at 6 hours compared with controls (Kruskal-Wallis: *P*=0.01; Dunn's: *P*=0.03 for predicted survivors, and *P*=0.05 for predicted non-survivors) (Figure 37). Levels subsequently decreased at 24 and 72 hours in septic rats, with no differences seen against sham controls. Corticosterone levels in septic animals at 6 hours exceeded this range, but returned closer to the normal range at later time-points (24 and 72 hours).

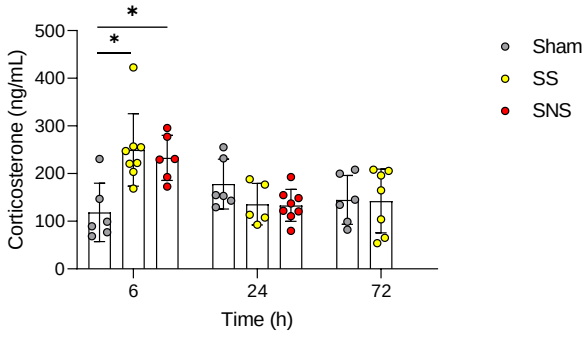

Figure 37. Serum levels of corticosterone. Corticosterone was measured by ELISA in serum samples taken at 6, 24 and 72 hours after catheterisation from rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. Data presented as mean ± SD. SS: Septic Survivor; SNS: Septic Non-Survivor; h: hour. **P*<0.05.

4.3.2.4 Cytokine measurements

Levels of the predominantly pro-inflammatory cytokine IL-6, and anti-inflammatory cytokine IL-10, were measured in serum from septic and sham-operated controls rats at the three pre-determined timepoints (6, 24 and 72 hours) (Figure 38).

At 6 hours, IL-6 and IL-10 cytokine levels were elevated in both septic groups. For IL-6, this was more marked in predicted non-survivors (Kruskal-Wallis: $P<0.0001$; Dunn's: $P=0.002$). During the established phase (24 hours), levels of IL-6 decreased in septic animals, making the difference with sham-operated control animals less pronounced. However, differences remained significant for septic non-survivors (Kruskal-Wallis: $P=0.02$; $P=0.03$). At the end of the experiment (72 hours), IL-6 levels in predicted survivors had completely returned to normal, with no difference between this group and sham-operated control animals.

During the early phase of sepsis (6 hours), predominantly anti-inflammatory cytokine IL-10 was elevated in both sepsis survivors (Kruskal-Wallis: $P=0.0003$; Dunn's: $P=0.003$) and non-survivors ($P=0.03$) compared to sham-operated controls, with no significant difference between the two septic groups. A similar trend was observed during the established phase (24 hours) (Kruskal-Wallis: $P=0.0003$), with differences between sepsis survivors ($P=0.03$) and non-survivors ($P=0.003$) compared to sham-operated control animals. During the recovery phase (72 hours), levels of IL-10 were still significantly higher in sepsis survivors ($P=0.001$).

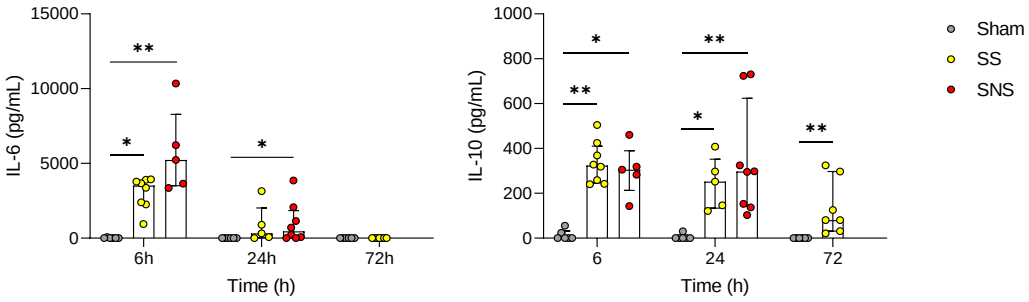

Figure 38. Serum levels of cytokines. Cytokines were measured by ELISA in serum samples taken at 6, 24 and 72 hours after catheterisation from rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. Data presented as mean ± SD. SS: Sepsis Survivor; SNS: Sepsis Non-Survivor; h: hour. *P<0.05; **P<0.01; ***P<0.001.

4.4 Discussion

In the current *in vivo* rat model of faecal peritonitis, I have replicated most endocrine, metabolic, and immune features previously described by my host lab. Early (6 hours) alterations in endocrine, metabolic, and immune profiles sometimes persisted through the established (24 hours) and recovery (72 hours) phases of sepsis. During the early phase (6 hours), before any other clinical signs of illness, mortality could be accurately predicted based upon differences in SV measured by echocardiography. However, for most other measurements, no differences were detected between predicted survivors and non-survivors.

4.4.1 Model severity

The severity of the current model resulted in a 72-hour mortality rate of 56%, which is similar to previous results in the lab using the same model [417], but slightly higher compared to mortality rates in human sepsis with faecal peritonitis [562]. I examined the impact of lower concentrations of faecal slurry (≤6 mL/kg) on mortality rates in an initial dose-titration study, but this resulted in mortality rates ≤16%, hence the choice

for a 7 mL/kg inject to resemble human faecal peritonitis and increase the chances of detecting differences in endocrine, metabolic, and immune parameters.

4.4.2 Thermoregulation

Body temperature in rats was not significantly different between groups at any given timepoint. During human sepsis, both fever and hypothermia are hallmark characteristics, with hypothermia being associated with adverse outcomes [565-567]. Early falls in core temperature and whole-body metabolism have repeatedly been found in murine models of sepsis [142], though not in rats. In previous work from the lab using the same faecal peritonitis rat model, either no difference [142] or an increase in temperature has been seen [417, 568].

This difference may be methodological in that the temperature control of the lab is suboptimal, often overheating in summer and cold in winter. The equilibrium point of heat production and loss, thermoneutrality, occurs at an ambient temperature of approximately 21°C in humans and 23-25°C in rats. Exposure to an environmental temperature either above or below thermoneutrality will trigger physiological responses that work to maintain a stable core body temperature. Ongoing work in the lab is assessing this question.

4.4.3 Haemodynamic stress

Early haemodynamic prognostication (6 hours) was performed on animals before they started to identify themselves clinically. Early myocardial depression and cardiac dysfunction were evident in septic animals despite fluid resuscitation. Septic animals showed an early decrease in SV and CO, and this was even more pronounced in the animals that died before the 72-hour study endpoint. Importantly, animals surviving 72 hours show clinical signs of improvement, so these can be considered true survivors rather than delayed deaths. This decrease in SV at 6 hours compared to

baseline values has proven to be an accurate predictor of mortality in the current model, and was subsequently used to assign mortality to rats. This is consistent with previous work in the lab using the same model [417, 563].

Substantial evidence of cardiac injury and myocardial depression has also been reported in a similar model, with more injury in non-surviving rats. These rats have a diminished ability to tolerate repeated fluid loading compared to predicted septic survivors and controls [563]. Early cardiac dysfunction in non-survivors was also manifested by tachycardia, raised serum troponin T, and B-type Natriuretic Peptide (BNP) [417].

4.4.4 O_2 delivery

The O_2 delivery (DO_2) was lower in predicted non-survivors compared to sham-operated controls, and fell below normal reference values for both predicted survivors and non-survivors during the early (6 hours) and established (24 hours) phases of sepsis. This fall was mainly caused by a decreased CO in septic rats at 6 hours, and low pO_2 and sO_2 levels in sepsis at 24 hours. The latter can probably be explained by the number of cardiac punctures that had to be performed at these timepoints in septic rats, resulting in a mix of arterial and venous blood. The Hb remained within normal range throughout the experiments in all groups, and was even higher in septic animals compared to sham-operated controls. This is likely to be due to haemoconcentration despite the considerable amount of fluid infused into these animals.

VO_2 was measured in rats housed in metabolic cages during the initial survival study. VO_2 fell immediately after faecal slurry injection both in sepsis survivors and non-survivors with loss of diurnal variation. Rates in sepsis survivors returned back to normal during the recovery phase of sepsis (24 hours). These DO_2 and VO_2 results are consistent with previous work in the lab with the same model [417].

Sufficient O_2 must be present in the cells to maintain adequate generation of ATP by the electron transport system. If O_2 availability is limited, ATP production is compromised and this results in a fall cellular O_2 consumption. In a very short term response this is pathological, but over time the cell may adapt to a persisting decrease in energy substrate availability by reducing its metabolism. Either way, this could lead to cellular and thus organ dysfunction. In concordance with the above findings, an increase in blood lactate concentrations was observed in septic rats at 24 and 72 hours, suggesting the initiation of a compensatory increase in glycolytic ATP production. Large trials have reported hyperlactataemia alone in 30-47% of patients with septic shock [569-571].

The interpretation of the blood lactate concentration is not straightforward [572]. Increased lactate concentrations could be caused by tissue hypoxia, especially before resuscitation, or by decreased clearance due to hepatic dysfunction. In my study, levels of bilirubin, an indicator of liver function, exceeded the normal range in all groups and at all time-points. Increased lactate could also be secondary to other mechanisms such as increased aerobic glycolysis or abnormal pyruvate metabolism [573]. On the other hand, lactate utilisation may increase in sepsis, as it is an important metabolic substrate for vital organs during stress states.

4.4.5 Metabolic alterations

Significant alterations occurred in plasma glucose and lactate levels during sepsis. Changes in lactate have been discussed in the previous paragraph. Glucose levels decreased in septic animals at 24 and 72 hours, without any differences between predicted survivors and non-survivors. Using RER metabolic data, assumptions can also be made on changes in substrate utilisation during sepsis.

Blood glucose levels were abnormally high in sham-operated control animals from the start. Although food intake was not monitored, this could potentially be explained by

continuous glucose administration to avoid hypoglycaemia. However, glucose has previously been administered in a similar model, without induction of hyperglycaemia [142]. Depleted glycogen stores, impaired gluconeogenesis, and increased peripheral glucose utilisation may all be contributing factors to the lower glucose levels seen in the septic rats.

Glucose levels during sepsis can be affected by stress hormones such as glucagon, growth hormone, catecholamines, and glucocorticoids, but also by pro-inflammatory cytokines [574]. Excessive insulin suppresses hepatic gluconeogenesis and regulates glucose uptake into skeletal muscle [59]. Insulin treatment for tight glycaemic control during sepsis has shown both benefit and harm [89].

Studying VO_2 consumption and VCO_2 production, and subsequently calculating correlating RER values, can give an insight into substrate utilisation. RER values fell in septic animals with a loss of diurnal rhythm. RER values during sepsis decreased to 0.75-0.8 without recovery, whereas they remained around 0.88-0.92 in sham-operated control animals. This suggests a change in metabolic substrate in septic animals away from carbohydrates towards a mix of fat and protein [575]. This switch in fuel utilisation is often seen in sick animals and during starvation and has also been reported in our septic rat model [142].

4.4.6 Endocrine changes

Both early and prolonged changes were found in levels of stress and metabolic hormones during the time-course of sepsis, without any differences between predicted survivors and non-survivors.

Levels of the metabolic hormone glucagon did not differ between the groups at any of the time-points. In a similar rat model of sepsis, levels of glucagon were initially elevated, with higher levels in non-survivors. Subsequently, levels returned to normal

range [417]. Similar findings to this have been found in human sepsis, where glucagon normally rises to oppose the role of insulin, with higher levels of glucagon in those who die [71, 96].

Contrary to circulating glucagon, insulin levels were significantly lower in septic animals at both 24 and 72 hours, without any differences between predicted survivors and non-survivors. Persistently repressed insulin levels in septic animals have been shown previously in the lab [417]. These results are also in accordance with an initial fall in insulin that is normally observed during critical illness [59]. The question remains whether this is due to suppressed secretion of insulin by the pancreas, or increased breakdown. During prolonged critical illness, hyperinsulinaemia and the development of insulin resistance is more common [59, 576].

Levels of the catecholamines, noradrenaline and adrenaline in both septic and sham animals exceeded reference values for healthy animals. At 6 hours, this may relate in part to a stress response following surgery. However, and surprisingly, there were no differences in levels between septic and sham-operated control animals at this time-point. Levels in sham-operated animals were even significantly higher at 24 hours. This contradicts findings from the literature and those previously found in a similar model [417]. A possible explanation is that the sham animals were excessively stressed but this was not apparent from their behaviour in the cage while on the tether. Early elevations in catecholamines have been reported during sepsis and subsequently decrease [66]. The magnitude of rise prognosticates for a poor outcome [342].

Similarly, unexpected results were obtained with thyroid hormone levels which, again, did not agree with previous measures made in this lab model and with the ‘sick euthyroid’ syndrome values found during sepsis [88, 90, 577]. In a similar rat model, lower levels of fT_3 were found in non-survivors [417]. In humans, a fall in thyroid hormones is prognostic in critical illness [89]. Significant differences between septic

and sham operated control rats were found for both fT_3 and rT_3 at 24 hours, with higher levels of fT_3 in septic animals and higher levels of rT_3 in sham operated control animals. However, this is contrary to what would be expected.

With thyroid hormones as key drivers of metabolism, an increase in fT_3 suggests increased metabolism during sepsis. The unexpected decrease in catecholamines in the current model and rise in fT_3 might be linked because of the common precursor tyrosine. An elevation in tyrosine iodination could be at the expense of catecholamine formation.

Lastly, a significant increase in corticosterone, the rat equivalent of cortisol, was found during the early phase (6 hours) in septic animals. A rise in corticosteroid hormones in critical illness has been attributed to reduced breakdown rather than increased production [81]. The pattern in rats differs from critically ill patients, where an initial increase of circulating cortisol is followed by a prolonged elevation [416, 578]. This initial elevation of corticosterone leads to a catabolic phase of metabolism. A subsequent fall in corticosterone suggests a move away from catabolism as recovery begins.

4.4.7 Immune response

The predominantly pro-inflammatory cytokine IL-6 increased during the early phase (6 hours) of sepsis, whereas the anti-inflammatory cytokine IL-10 remained elevated at all time-points, even during the recovery phase (72 hours). No differences were however found between predicted survivors and non-survivors for any of the cytokines measured.

Our previous lab studies in a similar rat model found a comparable trend in IL-6 levels, with a very early rise (3 hours) slowly coming down back to normal at 48 hours. Serum IL-10 peaked between the early (3 hours) and established phase (24 hours) and

declined thereafter, but remained elevated at 72 hours [568]. Higher levels were however found in non-survivors for IL-6 at 6 and 24 hours [417].

An early combined pro- and anti-inflammatory response is followed by a predominantly anti-inflammatory milieu, predisposing patients to secondary infections. Studies report elevated levels of IL-6 and IL-10 in septic patients on admission to critical care, with higher levels in those with septic shock and those who go on to die [579, 580]. High levels of these cytokines at discharge even correlated with increased mortality up to a year following admission [581].

4.5 Strengths and limitations

The aim of this *in vivo* rat study of faecal peritonitis was to characterise the endocrine, metabolic, and immune response during sepsis, and to examine differences in the stress response between survivors and non-survivors. Although this has largely been achieved, there are a number of limitations to the current work.

As with any critical illness, individual patient characteristics affect outcome. These characteristics include, but are not limited to, co-morbidities, interventions, age, and sex. I tried to adhere to the guidelines proposed by the MQTiPSS, to reflect human sepsis. I did not use antibiotics or organ support, and the homogenous group of previously healthy, male, adolescent rats obviously differ from the markedly variable human population treated for sepsis. Whereas patients present with sepsis after varying amounts of time, the timing of the infectious illness was precisely known in this rat model.

The number of rats used was kept to a minimum, due to animal welfare issues. Although the number used was based on previous experiments with the same model, this may have led to a type II error. Due to clotting of arterial lines during the study, no consecutive measurements in the same rats were possible. Therefore, separate

animals had to be used for the pre-determined timepoints, making it impossible to draw conclusions on trends over time.

An attempt was made to study the endocrine, metabolic, and immune response during sepsis. Although this study has included the most important markers of endocrine, metabolic, and immune function, more could have been included to study specific pathways. Animals (and humans) do lose circadian rhythms in sepsis so, ideally, sampling would have been performed at multiple time-points, but this is logistically challenging in view of the issues with arterial line occlusion.

4.6 Summary and conclusions

This 3-day fluid resuscitated rat model of faecal peritonitis displays early and wide-ranging changes in endocrine, metabolic, and immune markers, without many of the differences previously seen in this model between predicted survivors and non-survivors. Prognostication was still possible using SV, indicating the importance of myocardial injury/depression in determining outcome. Several markers remained abnormal, even after clinical recovery of the rats at the 72-hour study endpoint. This includes failure to restore the normal diurnal variation in VO_2 and VCO_2. It is likely that predicted survivors are developing a chronic stress response at this stage.

Conflicting results were found during the time course of sepsis, which are not in line with current understandings of the disease process, for example, stress hormone levels such as catecholamines and thyroid hormones. It is unclear why these were obtained as assay standard curves were excellent. The crucial question remains as to how the endocrine changes during sepsis affect mitochondrial and immune function, and as to whether altering the endocrine response would be beneficial for these parameters. It is reasonable to assume that an appropriate degree of endocrine changes is vital in dealing with an exogenous insult, yet an excessive response may be injurious.

Chapter 5 Summary, conclusion, and future ideas

In this thesis, I studied changes in various stress and metabolic hormones, and in markers of mitochondrial and immune function during sepsis. Subsequently, the impact of these endocrine changes in modulating mitochondrial and immune function of mononuclear cells were examined. Methods included *ex vivo* studies on patient samples, a rat model of faecal peritonitis, and *in vitro* studies with isolated human PBMCs.

5.1 Summary

Changes in some, but not all, stress and metabolic hormones were found, both in human sepsis and in the rat model of faecal peritonitis. An early increase in cortisol (and corticosterone in rats) was the most profound, but without any changes between survivors and non-survivors, despite accurate prognostication of survival using SV at 6 hours in the animal model. The persistent decrease in insulin levels in the rat model could not be replicated in humans. Surprisingly, both models did not show any changes in levels of catecholamines and glucagon. Where this was similar for both thyroid hormones in humans, increased fT_3 but decreased rT_3 were observed during the established phase of sepsis in the rat model.

Even though no changes in MMP were found in septic patients, there was a significant rise in mROS indicative of mitochondrial stress. Although cell-specific mitochondrial function was not assessed in the rat model, whole-body metabolism shifted during sepsis from carbohydrates towards the utilisation of protein and fat, and lost its diurnal rhythm. The rat model showed an early rise in both pro- and anti-inflammatory cytokines, with the latter even persisting through to the recovery phase. Changes in

cytokines were less profound in humans, but a decrease in quantified monocyte HLA-DR expression was evident.

As could be predicted, positive correlations were found between MMP and mROS in all groups of patients as the increase in protonmotive force generated by the higher membrane potential will drive ROS formation as a consequence. This reflects mitochondrial stress and/or a greater ATP demand. Of note, opposite directions of correlations were found between measures of mitochondrial and immune function in the different patient groups. While MMP was negatively correlated with monocyte HLA-DR in survivors, this correlation was positive in non-survivors. In control patients, mROS was positively correlated with monocyte HLA-DR. Whether this reflects an adaptive response in survivors requires further investigation.

Variable associations were seen between catecholamine levels and the various measures of mitochondrial and immune cell function in the control and patient groups. It is difficult, however, to see a consistent pattern when comparing either adrenaline or noradrenaline, or septic survivors and non-survivors. Subsequent *in vitro* studies did show that catecholamine-induced effects on mitochondrial and immune function are dose, stimulation, and cell-type specific. mROS and monocyte HLA-DR expression were seen to both increase and decrease depending on dose and cell type. Despite the increase in mROS, MMP often fell suggestive of damage/inhibition to the ETC. Similarly, the *in vitro* responses to cortisol were variable in terms of effects on immune function, and there was no measurable impact on mitochondria.

In non-survivors, positive correlations were found between both thyroid hormones (active fT_3 and inactive rT_3) and measures of mitochondrial function, HLA-DR expression, and both pro- and anti-inflammatory cytokines. The fact that both fT_3 and rT_3 show similar correlations argues against the reported metabolic inactivity of rT_3 in the literature. However, in survivors, an inverse relationship was seen between rT_3 and mROS. No correlations were found between insulin or glucagon and measures

of mitochondrial and immune function in most cases, except for a positive correlation between insulin and mROS in survivors.

5.2 Conclusion

In conclusion, although further studies are warranted to confirm and elaborate on the current observations, the opposite directions of correlations suggest a change in regulation of these processes in sepsis. Early identification of the processes that differ in those who go on to die, and also the description of how they differ, may benefit selecting optimal treatment strategies for patients. However, due to the nature of the current study, conclusions cannot be drawn on causation of correlations and caution therefore has to be applied interpreting these results.

The *in vitro* effects of catecholamines and cortisol differed per cell type, stimulation, and dose. This highlights again the importance of stratifying patients according to, for example, immune status, to identify who could benefit from elimination or administration of these hormones, or from manipulation of endocrine effects by using receptor blockers such as α- or β-blockers for catecholamines, or GR antagonists for glucocorticoids.

The relatively small sample sizes may have introduced Type II errors, though this would not explain the opposing correlations seen in, for example, survivors versus non-survivors. Previous execution of the rat model and comparison with human data by others in the lab revealed many more similarities in the endocrine response, and even prognostication of survival for some. I was unable to show this, even though both animals and patients looked clinically unwell, which was reflected by measures of, for example, lactate, cytokines, metabolism, and glucocorticoids. Although inter-operator variability is expected to some extent, this does not explain why changes are observed in some, but not all markers of the stress response during sepsis.

For consistency, I used similar kits and reagents to those of previous colleagues wherever possible, and adhered to the standardised protocols within the lab. New protocols were validated and optimised extensively, and included the use of controls, which indicated my techniques were methodologically sound. Drugs used during my *in vitro* experiments were freshly prepared and used before reaching the expiration dates.

Another potential explanatory factor is bad sample quality. To minimise this, I used liquid nitrogen to snap-freeze my samples from the animal experiments immediately after collection, which were subsequently stored at -80°C. Samples from septic patients were transported for processing and storage at -80°C as soon as possible after collection. Repeated thawing and freezing were prevented by storing samples in small batches for analysis. Errors in the methodology used or sample quality are therefore also unlikely explanatory factors, making I am unable to explain some of the unexpected results in the current thesis.

5.3 Future ideas

While I examined the impact of multiple hormones using patient samples, an *in vivo* rat model, and *in vitro* models of infection, I did not investigate underlying mechanisms, due in part to the lack of consistent findings that merited a deeper dive. Further studies are warranted to confirm observations and overcome abovementioned potential issues and type II errors that might have been encountered in the current study.

One obvious next step would be to focus on one hormone and cell type to provide us with mechanistic insights into the biological pathways underlying observed effects. For example, one could study hormone receptor involvement, subsequent effects of blocking these, and downstream signalling pathways. This would ideally include cells derived from patients, both survivors and non-survivors, to study distinct effects in

these patient groups and identify patients who would benefit from modulation of endocrine levels.

Including more measures of mitochondrial and immune cell function, such as respirometry and *in vitro* cytokine production, would help shape a more complete picture of effects. With these additional *in vitro* experiments, one must keep translational aspects in mind, using levels of hormones that could be physiologically encountered by cells during sepsis. Finally, animal studies are necessary due to the highly complex, heterogenous, and biphasic nature of the disease, and interplay between hormones. Our rat model of peritonitis could, for example, be used to unravel optimal timing and dosing strategies to improve outcomes, and hopefully translate these into the clinical setting.

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Appendix I – Greek ethics approval

ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ

ΔΙΟΙΚΗΣΗ 2ης ΥΓΕΙΟΝΟΜΙΚΗΣ ΠΕΡΙΦΕΡΕΙΑΣ ΠΕΙΡΑΙΩΣ ΚΑΙ ΑΙΓΑΙΟΥ

ΠΑΝΕΠΙΣΤΗΜΙΑΚΟ ΓΕΝΙΚΟ ΝΟΣΟΚΟΜΕΙΟ

«ΑΤΤΙΚΟΝ»

ΑΠΟΣΠΑΣΜΑ ΠΡΑΚΤΙΚΩΝ ΕΠΙΣΤΗΜΟΝΙΚΟΥ ΣΥΜΒΟΥΛΙΟΥ

5ης Συνεδρίασης

στις 18/06/08

ΑΠΟΦΑΣΗ

Θέμα : Έγκριση τροποποίησης του ερευνητικού πρωτοκόλλου «Μελέτη των μεταβολών της ανοσιακής απάντησης του ασθενούς κατά τη μετάβαση από σήψη σε σοβαρή σήψη και σηπτική καταπληξία» (Δ΄ Π.Π.Κ., 163/05-06-08)

Το Επιστημονικό Συμβούλιο (**παρόντες**: Καθηγητής Δημήτριος Κρεμαστινός, Αναπληρώτρια Καθηγήτρια Κυριακή Κανελλακοπούλου, Αναπληρώτρια Καθηγήτρια Γεωργία Γερολουκά – Κωστοπαναγιώτου, Λέκτορας Ιωάννης Κακίσης, Επίκουρη Καθηγήτρια Κλεάνθη Δήμα, Επίκουρος Καθηγητής Παναγιώτης Χαλβατσιώτης, Ειδικευόμενη Ιατρός Αικατερίνη Δημουλά, Τομεάρχης Νικόλαος Γράσσος) αφού έλαβε υπόψη του:

1) την αριθμ. 5/18-06-08 απόφαση της Επιτροπής Βιοηθικής Δεοντολογίας (θέμα 21°) (**Παρόντες**: Αναπληρώτρια Καθηγήτρια Κυριακή Κανελλακοπούλου, Αναπληρώτρια Καθηγήτρια Γεωργία Γερολουκά – Κωστοπαναγιώτου, Αναπληρώτρια Διευθύντρια Αθανασία Τζιώκα, Επίκουρος Καθηγητής Παναγιώτης Χαλβατσιώτης, Λέκτορας Ιωάννης Κακίσης)
2) τις διαδικασίες και τους κανονισμούς υποβολής πρωτοκόλλων για διενέργεια κλινικής δοκιμής στο Νοσοκομείο

3) το γεγονός ότι οι φάκελοι είναι πλήρεις και καλύπτουν όλες τις προϋποθέσεις για τη διεξαγωγή της μελέτης

4) ότι από τη διενέργεια της μελέτης δεν προκύπτει οικονομική επιβάρυνση για το Νοσοκομείο

Ομόφωνα Αποφασίζει

Έλαβε γνώση αναφορικά με την τροποποίηση του ερευνητικού πρωτοκόλλου «Μελέτη των μεταβολών της ανοσιακής απάντησης του ασθενούς κατά τη μετάβαση από σήψη σε σοβαρή σήψη και σηπτική καταπληξία», που διεξάγεται στη Δ΄ Π.Π.Κ.

Παραπέμπει το θέμα στο Δ/κό Συμβούλιο για ενημέρωση και περαιτέρω έγκριση.

Ο Πρόεδρος του Επιστημονικού Συμβουλίου

Καθηγητής Δημήτριος Κρεμαστινός

Appendix II – Participant information and consent

Τίτλος Πρωτοκόλλου: Μελέτη Προσδιορισμού των Μεταβολών της Ανοσιακής Απάντησης του Ασθενούς κατά τη Μετάβαση από Σήψη σε Σοβαρή Σήψη και Σηπτική Καταπληξία

Αρχικά ασθενούς:_____ Αριθμός ασθενούς:_____

Όνομα ερευνητή:_____

ΠΛΗΡΟΦΟΡΙΑΚΟ ΣΗΜΕΙΩΜΑ / ΔΗΛΩΣΗ ΣΥΓΚΑΤΑΘΕΣΗΣ

ΕΙΣΑΓΩΓΗ-ΣΚΟΠΟΣ ΤΗΣ ΜΕΛΕΤΗΣ

Σας ζητείται η συγκατάθεση για τη συμμετοχή σας σε μία ερευνητική μελέτη. Θα σας ζητηθεί να διαβάσετε αυτό το έντυπο συγκατάθεσης και να συζητήσετε οτιδήποτε δεν καταλαβαίνετε με τον γιατρό της μελέτης ή το ιατρικό προσωπικό. Εφόσον κατανοήσετε τη μελέτη θα σας ζητηθεί να υπογράψετε και να βάλετε ημερομηνία σε αυτό το έντυπο συγκατάθεσης σε περίπτωση που επιλέξετε να συμμετέχετε στη μελέτη. Θα σας δοθεί αντίγραφο του υπογεγραμμένου εντύπου συγκατάθεσης.

Μπορείτε να λάβετε μέρος ή να διακόψετε τη συμμετοχή σας στη μελέτη σε οποιαδήποτε στιγμή εφόσον το αποφασίσετε εσείς χωρίς να χάσετε το πλεονέκτημα της συνηθισμένης ιατρικής φροντίδας που λαμβάνετε έως τώρα.

Αν το επιθυμείτε, ο γιατρός που σας παρακολουθεί θα επικοινωνήσει με τον οικογενειακό σας γιατρό, προκειμένου να τον ενημερώσει για τη συμμετοχή σας στη μελέτη.

Οι σοβαρές βακτηριακές λοιμώξεις σε ορισμένες περιπτώσεις επιπλέκονται με σηπτικό σύνδρομο. Πρόκειται για βαρειά κλινική εικόνα που σχετίζεται με αυξημένο κίνδυνο θανάτου ιδιαίτερα αν εξελιχθεί σε σηπτική καταπληξία και ανεπάρκεια πολλών οργάνων.

Σκοπός της παρούσας μελέτης είναι να περιγραφούν με ακρίβεια οι παράγοντες που οδηγούν στην εξέλιξη από τη σήψη στη σοβαρή σήψη, στη σηπτική καταπληξία και στην ανεπάρκεια πολλών οργάνων. Οι συλλεγείσες πληροφορίες θα αναλυθούν με σκοπό να εξαχθούν συμπεράσματα για τα κάτωθι: α) τους θεραπευτικούς χειρισμούς για τους ασθενείς, β) τους πολυμορφισμούς του DNA των ασθενών , και γ) τις μεταβολές της λειτουργίας του ανοσιακού μηχανισμού που παρατηρούνται στο σηπτικό σύνδρομο.

ΣΧΕΔΙΟ ΤΗΣ ΜΕΛΕΤΗΣ ΚΑΙ ΔΙΑΔΙΚΑΣΙΕΣ

Οι παρακάτω εξετάσεις και διαδικασίες θα πραγματοποιηθούν ώστε να διαπιστωθεί αν μπορείτε να συμμετέχετε σε αυτή τη μελέτη:

- Θα επιθεωρηθεί το ιατρικό σας ιστορικό, προηγούμενη και παρούσα χρήση φαρμάκων.
- Αξιολόγηση της παρούσας κατάστασής σας με πλήρη φυσική εξέταση και διαγνωστικό έλεγχο που θα συμπεριλαμβάνει δείγματα αίματος.

Έκδοση 07/05/2018

Αρχικά ασθενούς:_____ Αριθμός ασθενούς:_____

Όνομα ερευνητή:_____

Θα παρακολουθείσθε για 28 ημέρες και η εκτίμηση της κατάστασης σας θα βασίζεται σε κλινικά κριτήρια και σε εργαστηριακά δεδομένα. Δείγμα αίματος για τη μελέτη θα ληφθεί και σε περίπτωση μεταβολής της κλινικής σας κατάστασης.

Ποσότητα 12ml αίματος θα ληφθεί από εσάς υπό άσηπτες συνθήκες. Το αίμα θα μοιρασθεί σε 3 σωληνάρια; 1) ένα με 3ml για ανάλυση του DNA 2) ένα με 5ml για ανάλυση του RNA, και 3) ένα με 4ml για ανοσολογικές παραμέτρους. Τα σωληνάρια μεταφέρονται σε ένα κεντρικό εργαστήριο και αναλύονται ως ακολούθως:

- Το αίμα που συλλέγεται στο σωληνάριο (1) χρησιμοποιείται για την απομόνωση του DNA. Το DNA είναι το μόριο που έχει όλες τις γενετικές μας πληροφορίες. Αυτό «αποφασίζει» πώς αντιδρά το σώμα σας και πώς γεννιέται η σήψη. Πιστεύουμε ότι το DNA σας μπορεί να φέρει κάποια ειδική μετάλλαξη ή πολυμορφισμό που είτε αυξάνει είτε μειώνει τον κίνδυνό σας να αναπτύξετε σήψη ή που επηρεάζει την τελική έκβαση του νοσήματός σας. Το DNA σας θα αναλυθεί για να προσδιορισθούν αυτές οι μεταλλάξεις ή πολυμορφισμοί.
- Το αίμα που συλλέγεται στο σωληνάριο (2) χρησιμοποιείται για την απομόνωση του RNA. Το RNA είναι το μόριο που φέρει εκείνα τα γονίδια που είναι έτοιμα να παράγουν πρωτεΐνες. Μερικές από αυτές τις πρωτεΐνες συνδέονται με το νόσημά σας. Πιστεύουμε ότι η παρουσία αυτών των πρωτεϊνών σηματοδοτεί κάποιες αλλαγές που συμβαίνουν στο RNA σας. Συνεπώς το RNA σας θα αναλυθεί για να βρεθεί πώς αυτά τα γονίδια τροποποιούνται στο σώμα σας.
- Το αίμα που συλλέγεται στο σωληνάριο (3) χρησιμοποιείται για την απομόνωση του ορού. Ο ορός είναι ένα υγρό στο οποίο βρίσκονται όλες οι πρωτεΐνες και οι μεταβολίτες του σώματός μας. Ο ορός θα αναλυθεί για να βρεθεί πώς τα συστατικά του αλλάζουν και πώς αυτές οι αλλαγές δίνουν πληροφορίες για το νόσημά σας.

Η ανάλυση των δειγμάτων (1), (2) ή (3) μπορεί να γίνει σε κάποιο εργαστήριο στην Ελλάδα ή στο εξωτερικό κάτι το οποίο θα αποφασισθεί από τους ερευνητές.

ΠΙΘΑΝΟΙ ΚΙΝΔΥΝΟΙ Η ΠΑΡΕΝΕΡΓΕΙΕΣ

Η λήψη των δειγμάτων αίματος μπορεί να προκαλέσει μια προσωρινή ενόχληση από το τσίμπημα της βελόνας, όπως πόνο, αιμορραγία και/ή μώλωπα στη συγκεκριμένη περιοχή.

Τίτλος Πρωτοκόλλου: Μελέτη Προσδιορισμού των Μεταβολών της Ανοσιακής Απάντησης του Ασθενούς κατά τη Μετάβαση από Σήψη σε Σοβαρή Σήψη και Σηπτική Καταπληξία

Αρχικά ασθενούς:_____ Αριθμός ασθενούς:_____

Όνομα ερευνητή:_____

ΣΥΜΜΕΤΟΧΗ/ΔΙΑΚΟΠΗ ΣΥΜΜΕΤΟΧΗΣ ΣΤΗ ΜΕΛΕΤΗ

Η συμμετοχή σας σε αυτή τη μελέτη είναι εξ' ολοκλήρου εθελοντική και μπορείτε να αρνηθείτε τη συμμετοχή σας σε οποιαδήποτε στιγμή.

Ο γιατρός μπορεί να διακόψει τη συμμετοχή σας σε αυτή τη μελέτη χωρίς την συγκατάθεσή σας.

ΠΡΟΣΒΑΣΗ ΣΤΟΝ ΙΑΤΡΙΚΟ ΦΑΚΕΛΟ, ΠΡΟΣΤΑΣΙΑ ΤΩΝ ΠΡΟΣΩΠΙΚΩΝ ΔΕΔΟΜΕΝΩΝ

Ο ιατρός της μελέτης και η ερευνητική ομάδα θα συλλέξουν, θα καταγράψουν και θα χρησιμοποιήσουν προσωπικές σας πληροφορίες για τους σκοπούς της μελέτης. Ζητάμε την άδειά σας για συλλογή, χρήση και κοινοποίηση των Προσωπικών Δεδομένων που σας αφορούν όπως εξηγείται παρακάτω σε αυτή την ενότητα. Εάν δεν συμφωνείτε, δεν θα μπορείτε να συμμετάσχετε στη μελέτη. Οι προσωπικές πληροφορίες που θα συλλεχθούν κατά της διάρκεια της μελέτης ενδέχεται να περιλαμβάνουν προσωπικά στοιχεία (π.χ. όνομα, ηλικία, κτλ), ευαίσθητες πληροφορίες σχετικά με τη σωματική ή ψυχική σας υγεία ή κατάσταση, καθώς και πληροφορίες για την υγεία σας οι οποίες περιλαμβάνονται στους ιατρικούς φακέλους. Τα δεδομένα προσωπικού χαρακτήρα που σχετίζονται με εσάς προστατεύονται από την ελληνική και την ευρωπαϊκή νομοθεσία (ν. 2472/1997 «Προστασία του ατόμου από την επεξεργασία δεδομένων προσωπικού χαρακτήρα με ενσωματωμένες τις τροποποιήσεις» ΦΕΚ 50/Α/10.04.1997 και Κανονισμός (ΕΕ) 2016/679). Όλα τα δεδομένα που θα συλλεχθούν θα φυλαχθούν ώστε να προστατεύεται το ιατρικό απόρρητο για χρονικό διάστημα ίσο με τριάντα έτη βάση της ελληνικής και ευρωπαικής νομοθεσίας. Ο Υπεύθυνος Επεξεργασίας δύναται να διαβιβάσει δεδομένα προσωπικού χαρακτήρα σε χώρες και διεθνείς οργανισμούς εντός Ευρωπαϊκής Ένωσης. Ως Υπεύθυνος Προστασίας Δεδομένων του Χορηγού (DPO) ορίζεται η κ. Λήδα Ευστρατίου, τηλ επικοινωνίας: 210 7480662. Το όνομά σας θα αναφέρεται μόνο με τα αρχικά και έναν κωδικό αριθμό. Έχετε το δικαίωμα να αποκτήσετε πρόσβαση και να διορθώσετε τα προσωπικά σας δεδομένα, να περιορίσετε ή να εναντιωθείτε στην επεξεργασία τους, έχετε δυνατότητα μεταφοράς τους καθώς και διαγραφής τους. Σε περίπτωση παραβίασης των προσωπικών σας δεδομένων θα πραγματοποιηθεί ανακοίνωση στο άτομο σας. Μόνο το ιατρικό ερευνητικό προσωπικό μπορεί να έχει πρόσβαση στα στοιχεία αυτά. Το ιατρικό προσωπικό δικαιούται να παρουσιάσει τα δεδομένα που αφορούν την πορεία σας σε αρμόδια πρόσωπα που θα πιστοποιήσουν την ομαλή

Αρχικά ασθενούς:_____ Αριθμός ασθενούς:_____

Όνομα ερευνητή:_____

διεξαγωγή της μελέτης. Πρόσβαση στα στοιχεία της ταυτότητας σας μπορεί και να ζητηθεί για την εξασφάλιση της καλής διενέργειας της μελέτης καθώς και για την ανάλυση των δεδομένων από το χορηγό και τους εκπροσώπους αυτού (επιτηρητές μελέτης) καθώς και από τις αρμόδιες αρχές Ηθικής και Δεοντολογίας της χώρας (ΕΟΦ ή/και άλλη αρμόδια υπηρεσία). Οποιοσδήποτε από τους ανωτέρω έχει πρόσβαση στις πληροφορίες υποχρεώνεται από το νόμο να τηρήσει το ιατρικό απόρρητο. Τα δείγματα αίματος επίσης θα φέρουν κωδικό μελέτης και όχι το ονοματεπώνυμο σας ακριβώς όπως και οι κλινικές πληροφορίες που θα συλλεχθούν. Δεν θα υπάρξει δημοσίευση ή ανακοίνωση των δεδομένων αυτών που να αποκαλύπτουν την ταυτότητά σας. Σε περίπτωση που το επιθυμείτε έχετε το δικαίωμα υποβολής καταγγελίας στην Αρχή Προστασίας Δεδομένων Προσωπικού Χαρακτήρα (ΑΠΔΠΧ).

ΣΥΓΚΑΤΑΘΕΣΗ ΤΩΝ ΣΥΜΜΕΤΕΧΟΝΤΩΝ ΣΤΗ ΜΕΛΕΤΗ

Διάβασα το πλήρες κείμενο αυτού του εγγράφου και όλες μου οι απορίες, που αφορούν στη συμμετοχή μου στη μελέτη έχουν απαντηθεί. Κατανοώ ότι η συμμετοχή μου στη μελέτη είναι εθελοντική. Συμφωνώ με την απόδοση των ιατρικών μου πληροφοριών στους ερευνητές και στους αρμόδιους φορείς π.χ. την Επιτροπή Ιατρικής Δεοντολογίας του Νοσοκομείου, την Εθνική Επιτροπή Ηθικής και Δεοντολογίας και τον Ελληνικό Εθνικό Οργανισμό Φαρμάκων. Έχω ενημερωθεί για την προστασία Προσωπικών Δεδομένων που με αφορούν και εξουσιοδοτώ την πρόσβαση, χρήση και μεταφορά των Προσωπικών Δεδομένων μου και των βιολογικών δειγμάτων όπως περιγράφεται παραπάνω. Είμαι ελεύθερος να αποσυρθώ από τη μελέτη οποιαδήποτε στιγμή, για οποιοδήποτε λόγο και κατανοώ πως η απόφασή μου αυτή δεν θα επηρεάσει την μελλοντική μου ιατρική θεραπεία. Κατανοώ ότι θα λάβω αντίγραφο του υπογεγραμμένου εγγράφου, με την ημερομηνία υπογραφής. Υπογράφοντας αυτό το έγγραφο δεν παραιτούμαι από τα δικαιώματα μου ως μέτοχος μίας κλινικής μελέτης.

Συμφωνώ να συμμετέχω στο Πρωτόκολλο της Μελέτης: «Μελέτη Προσδιορισμού των Μεταβολών της Ανοσιακής Απάντησης του Ασθενούς κατά τη Μετάβαση από Σήψη σε Σοβαρή Σήψη και Σηπτική Καταπληξία»

Έκδοση 07/05/2018

Τίτλος Πρωτοκόλλου: Μελέτη Προσδιορισμού των Μεταβολών της Ανοσιακής Απάντησης του Ασθενούς κατά τη Μετάβαση από Σήψη σε Σοβαρή Σήψη και Σηπτική Καταπληξία

Αρχικά ασθενούς:_____ Αριθμός ασθενούς:_____

Όνομα ερευνητή:_____

Όνομα συμμετέχοντα στη μελέτη

Υπογραφή συμμετέχοντα στη μελέτη ή νόμιμου εκπροσώπου Ημερομηνία

Όνομα ιατρού που έλαβε τη συγκατάθεση μετά από ενημέρωση

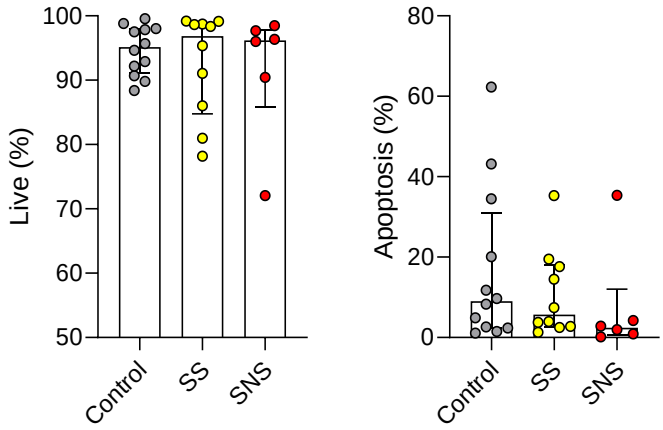
Υπογραφή ιατρού που έλαβε τη συγκατάθεση μετά από ενημέρωση Ημερομηνία

Έκδοση 07/05/2018

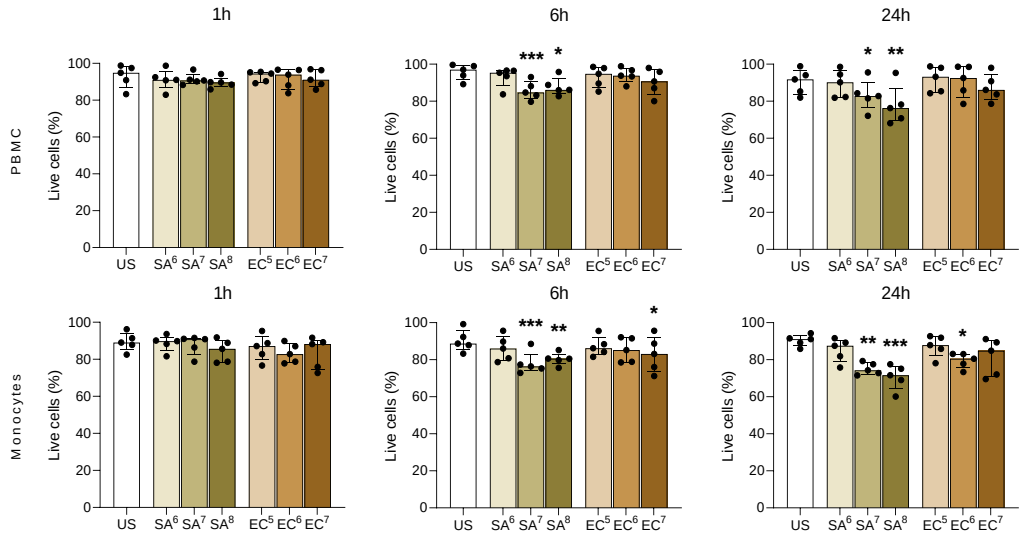
Appendix III – Supplementary data

Supplementary Table 1. Biochemical analyses of blood samples on Day 1. Blood was collected from patients with sepsis on the first day after admission. Patients are divided into survivors (SS) and non-survivors (SNS). Data presented as mean ± SD or median (IQR). SS: Sepsis Survivor; SNS: Sepsis Non-Survivor; NR: Normal Range; m: male; f: female.

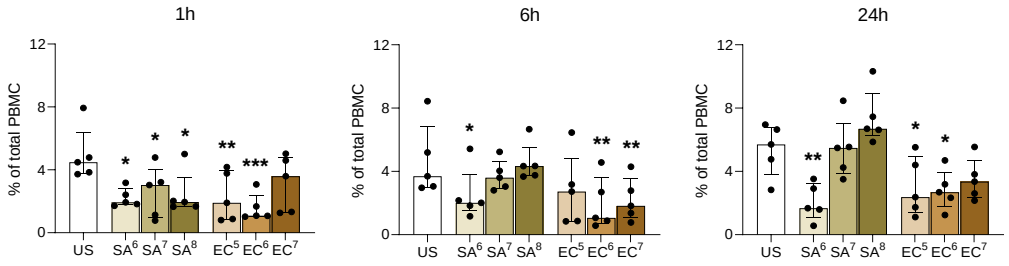
	SS (*n*=10)	**SNS (*n*=7)**	***P*-value**
pO_2 (mmHg) NR: 75-100	95.9 ± 45.6	120.3 ± 43.8	0.37
pO_2/FiO_2 (mmHg) NR: 400-500	323.8 ± 125.3	321.1 ± 118.1	0.97
pCO_2 (mmHg) NR: 35-45	33.0 ± 12.4	31.6 ± 6.6	0.82
pH NR: 7.35-7.45	7.40 ± 0.12	7.38 ± 0.13	0.78
HCO_3^- (mEq/L) NR: 22-28	22.6 ± 9.5	19.6 ± 6.7	0.56
Glucose (mg/dL) NR: 72-108	108.0 (72.5-189.5)	232.0 (160.0-234.0)	0.10
Lactate (mmol/L) NR: 0.5-2.2	1.10 (0.90-1.30)	2.0 (0.80-3.45)	0.50
Platelets (/uL) **N**R: 150,000-400,000	228,455 ± 121,673	154,800 ± 53,035	0.22
WBC (/uL) NR: 4,000-11,000	13,696 ± 4,624	14,908 ± 6,773	0.68
Bilirubin (mg/dL) NR: 0.29-1.23	0.68 ± 0.29	0.67 ± 0.62	0.43
Creatinine (mg/dL) NR *m*: 0.72-1.18 NR *f*: 0.55-1.02	0.80 (0.70-2.40)	1.60 (1.00-5.15)	0.36
Hb (g/dL) NR *m*: 13.5-18.0 NR *f*: 11.5-16.0	11.0 ± 2.2	11.8 ± 4.1	0.62



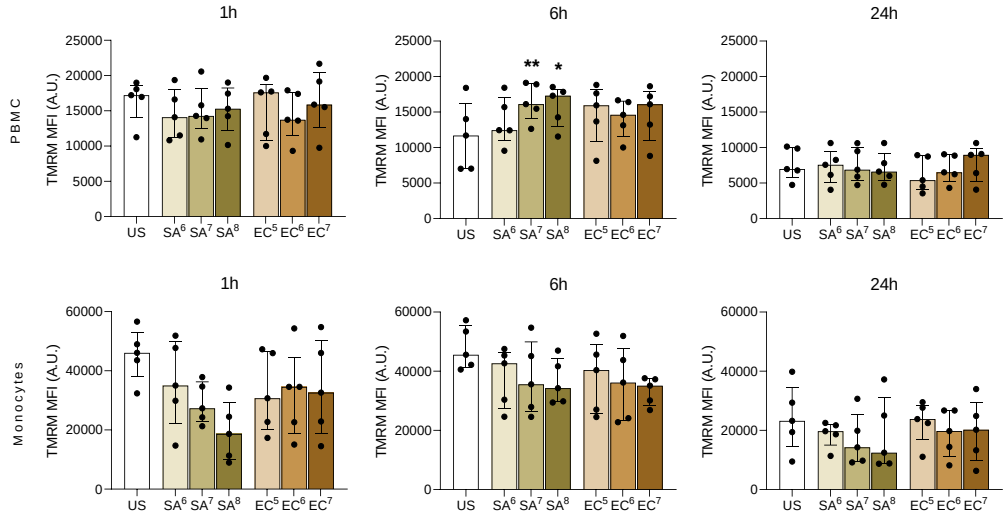
Supplementary Figure 1. Percentage of live and apoptotic monocytes Day 1. The percentage of live ($CD14^+/7AAD^-$) and apoptotic ($CD14^+/7AAD^-/Annexin\text{-}V^+$) monocytes was measured by flow cytometry of lysed whole blood samples from patients with sepsis (divided into survivors (SS) and non-survivors (SNS)) and pre-operative control patients on the 1st day after admission. Data presented as median ± IQR. SS: Septic Survivor; SNS: Septic Non-Survivor.



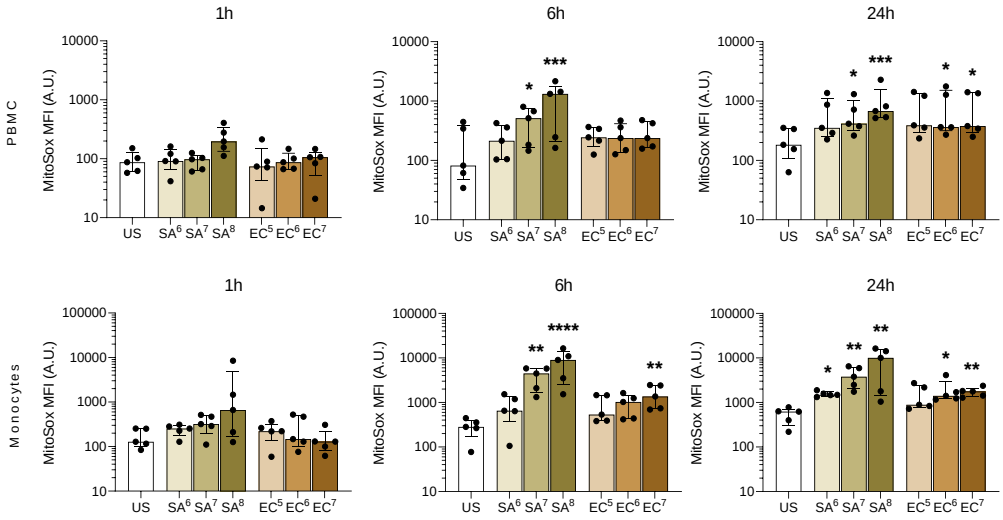
Supplementary Figure 2. Viability of PBMCs and monocytes after incubation with HKB (SA or EC). Isolated PBMCs from healthy volunteers (*n*=5) were incubated for 1, 6 and 24 hours with three different concentrations of two different HKB, being SA and EC. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocytes. The number of live (LIVE/DEAD$^-$) PBMCs and monocytes are expressed as percentage of the total amount of these cells. Data presented as median ± IQR. US: Unstimulated; SA: S. Aureus; EC: E. Coli. **P*<0.05; ***P*<0.01; ****P*<0.001 compared to US control cells.



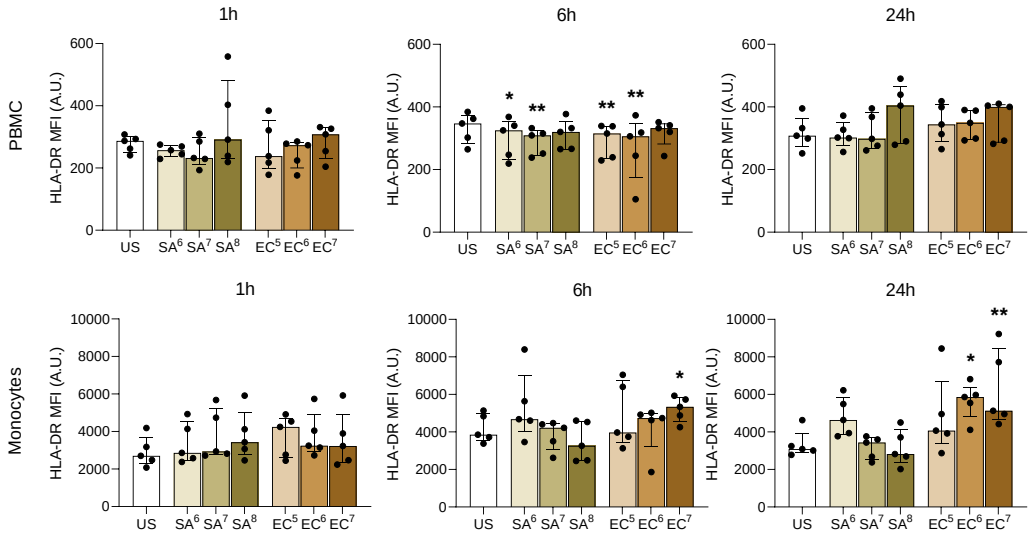
Supplementary Figure 3. Percentage of monocytes after incubation with HKB (SA or EC). Isolated PBMCs from healthy volunteers (*n*=5) were incubated for 1, 6 and 24 hours with three different concentrations of two different HKB, being SA and EC. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocytes. The number of monocytes is expressed as percentage of the total amount PBMCs in the sample. Data presented as median ± IQR. US: Unstimulated; SA: S. Aureus; EC: E. Coli. **P*<0.05; ***P*<0.01; ****P*<0.001 compared to US control cells.



Supplementary Figure 4. TMRM signal in live PBMCs and monocytes after incubation with HKB (SA or EC). Isolated PBMCs from healthy volunteers (*n*=5) were incubated for 1, 6 and 24 hours with three different concentrations of two different HKB, being SA and EC. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocytes. TMRM (measuring MMP) signal was measured in live monocytes (CD14/CD16^{+} and HLA-DR^{+}) only. Data presented as median ± IQR. MFI: Mean Fluorescent Intensity; A.U.: Arbitrary Units; US: Unstimulated; SA: S. Aureus; EC: E. Coli. **P*<0.05; ***P*<0.01 compared to US control cells.



Supplementary Figure 5. MitoSox signal in live PBMCs and monocytes after incubation with HKB (SA or EC). Isolated PBMCs from healthy volunteers (*n*=5) were incubated for 1, 6 and 24 hours with three different concentrations of two different HKB, being SA and EC. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocytes. MitoSox (measuring mROS) signal was measured in live monocytes (CD14/CD16^{+} and HLA-DR^{+}) only. Data presented on a log-scale as median ± IQR. MFI: Mean Fluorescent Intensity; A.U.: Arbitrary Units; US: Unstimulated; SA: S. Aureus; EC: E. Coli. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 compared to US control cells.



Supplementary Figure 6. HLA-DR on live PBMCs and monocytes after incubation with HKB (SA or EC). Isolated PBMCs from healthy volunteers (*n*=5) were incubated for 1, 6 and 24 hours with three different concentrations of two different HKB, being SA and EC. These samples were analysed by flow cytometry using pan-leukocyte marker CD45 for identification of PBMCs, and CD14/CD16 and HLA-DR for identification of monocytes. HLA-DR signal was measured in live monocytes (CD14/CD16$^+$ and HLA-DR$^+$) only. Data presented as median ± IQR. MFI: Mean Fluorescent Intensity; A.U.: Arbitrary Units; US: Unstimulated; SA: S. Aureus; EC: E. Coli. **P*<0.05 and ***P*<0.01 compared to US control cells.

Supplementary Table 2. Clinical severity scoring sheet. Animals were monitored at least 4 times a day during the experiment to assess characteristics such as appearance, behaviour, clinical signs, and other observations. MAP: Mean Arterial Pressure.

Score	**Body weight loss**
0	Normal <5%
1	5-10%
2	10-15%
3	>15-20%
Score	**Appearance**
0	Glossy coat, bright open eyes
1	Dull coat, slight piloerection, slight hunched, squinting or occasionally closed eyes
2	Ungroomed coat, piloerection, hunched, persistently closed eyes, dehydration, porphyrin staining
3	Soiled coat, piloerection, hunched, continuously closed eyes/discharge
Score	**Behaviour**
0	Alert and interested in the environment
1	Alert, occasionally interested in the environment
2	Depressed, little interest in the environment
3	Immobile, unresponsive
Score	**Clinical signs**
0	Normal temperature, cardiovascular and respiratory function
1	Warm to touch, MAP >90 mmHg, slight panting
2	Cold to touch, MAP 75-90 mmHg, laboured breathing
3	Cold to touch, MAP <75 mmHg, abdominal breathing
Score	**Other observations**
4	Chronic diarrhoea (over 48 hours), coagulopathy (blood in urine, mouth, faeces)
4	Pale mucous membranes
4	Paralysis, ataxia, convulsions
4	Vocalisation
4	Large/ulcerated solid mass, untreatable skin wounds

Supplementary Table 2. *Continued.*

Score	Action
0	Normal
1	Increase monitoring (minimum twice a day), give additional support (mashed food, etc.)
2	Increase monitoring (minimum 4 times a day), provide additional support (mashed food, fluids, additional nesting), cull if no improvement in 48 hours – (8 hours if moderate severity study)
3	Critically ill animal, increase monitoring (minimum every 4 hours), provide additional support (mashed food, fluids, nesting), continue monitoring overnight, or cull if no improvement at the at the end of the day – (immediate euthanasia if moderate severity study)
4	Immediate euthanasia

Supplementary Table 3. Microbiological analysis of faecal slurry. This analysis was performed by the UCLH Clinical Microbiology Lab on aliquots of faecal slurry prepared in 2018, and compared to analysis of faecal slurry from 2013.

2013		**2018**	
Bacteria	**CFU/mL**	**Bacteria**	**CFU/mL**
Aerobic		Aerobic	
Total viable count	$1.39x10^4$	Total viable count	$2.75x10^4$
Escherichia coli	$2.3x10^3$	*Escherichia coli*	$2.59x10^4$
Enterococcus thailandicus	$5.0x10^3$	*Hofnia alvei*	$1.6x10^3$
Enterococcus faecium	$5.5x10^3$		
Leuconostoc lactis	$1.1x10^3$		
Anaerobic		Anaerobic	
Total viable count	$1.8x10^6$	Total viable count	$7.3x10^6$
Collinsella aerofaciens	$1.0x10^6$	*Bacteroides ovatus*	$1.2x10^6$
Bifidobacterium longum	$8.0x10^6$	*Bacteroides uniformis*	$2.5x10^6$
		Bifidobacterium longum	$3.6x10^6$

Supplementary Table 4. Blood gas analysis. Blood was collected at 6, 24 and 72 hours after catheterisation from rats with sepsis (divided into survivors (SS) and non-survivors (SNS)) and sham control rats. Data presented as mean ± SD. NR: Normal Range; SS: Sepsis Survivor; SNS: Sepsis Non-Survivor; h: hour. **P*<0.05; ***P*<0.01; ****P*<0.001; *P*<0.0001 vs sham. $^{++}$*P*<0.01 vs SS.

		Sham	**SS**	**SNS**	***P*-value**
pH NR: 7.38-7.42	**6h**	7.48 ± 0.14	7.47 ± 0.15	7.44 ± 0.04	0.89
	24h	7.46 ± 0.05	7.39 ± 0.11	7.32 ± 0.09*	0.02
	72h	7.50 ± 0.04	7.45 ± 0.05		0.12
pO_2 (kPa) NR: 10.2-12.4	**6h**	10.8 ± 4.4	12.7 ± 4.8	10.8 ± 2.2	0.63
	24h	11.8 ± 3.2	10.9 ± 8.9	7.1 ± 3.6	0.20
	72h	11.0 ± 1.0	12.4 ± 1.7		0.13
pCO_2 (kPa) NR: 4.7-6.0	**6h**	5.4 ± 2.2	5.4 ± 1.4	4.6 ± 0.5	0.65
	24h	5.4 ± 0.5	7.0 ± 2.3	7.7 ± 2.4	0.13
	72h	5.0 ± 0.5	5.0 ± 0.9		0.96
HCO_3^- (mmol/L) NR: 22-28	**6h**	27.9 ± 1.4	25.5 ± 1.6*	24.4 ± 0.9**	0.003
	24h	28.6 ± 3.0	26.6 ± 1.6	24.5 ± 1.6**	0.009
	72h	29.0 ± 1.1	25.6 ± 1.7**		0.004
Base excess (mmol/L) NR: -2 - +2	**6h**	4.3 ± 3.2	1.9 ± 1.4	-0.3 ± 1.1**	0.008
	24h	5.4 ± 3.4	4.7 ± 2.1	2.7 ± 1.9	0.18
	72h	5.5 ± 1.2	1.4 ± 2.1**		0.004
K^+ (mmol/L) NR: 3.8-5.5	**6h**	4.6 ± 0.5	5.5 ± 0.4*	5.5 ± 0.5*	0.007
	24h	4.2 ± 0.4	4.7 ± 0.6	5.0 ± 1.0	0.14
	72h	4.3 ± 0.3	4.5 ± 0.4		0.21
Na^+ (mmol/L) NR: 140-146	**6h**	141 ± 1	135 ± 2*	133 ± 1**	0.0004
	24h	142 ± 2	142 ± 1	142 ± 5	0.82
	72h	140 ± 2	141 ± 1		0.49
Ca^{2+} (mmol/L) NR: 0.84-1.21	**6h**	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.02	0.07
	24h	1.2 ± 0.7	1.2 ± 0.1	1.1 ± 0.1	0.60
	72h	1.1 ± 0.1	1.2 ± 0.04*		0.02
Cl^- (mmol/L) NR: 99-106	**6h**	105 ± 8	101 ± 3	102 ± 2	0.66
	24h	102 ± 2	100 ± 4	102 ± 4	0.68
	72h	103 ± 1	104 ± 1		0.13

Supplementary Table 4. *Continued.*

		Sham	**SS**	**SNS**	***P*-value**
Bilirubin (umol/L) NR: 3-12	**6h**	38.5 ± 11.5	47.4 ± 6.3	51.0 ± 12.4	0.18
	24h	38.2 ± 8.1	31.8 ± 15.8	36.5 ± 9.9	0.63
	72h	35.3 ± 3.6	38.3 ± 11.0		0.55