Fatty acids on the crossroads of immune and metabolic pathways in sepsis

A thesis submitted to University College London for the

Degree of Doctor of Philosophy

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March 2021

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I, Vera Peters, 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 represents life-threatening organ dysfunction caused by a dysregulated host response to infection. Sepsis comprises various derangements including metabolic, immune and mitochondrial dysfunction and these are likely to be inter-related. The pharmacological impact of nutrition in sepsis is mostly overlooked. Depending on their hydrocarbon chain lengths, degree of unsaturation (number of double bonds between carbon atoms), number, position and orientation of their double bonds, lipids have differential effects on immune function and metabolism to either the potential benefit or detriment the patient.

Objectives: This study examines the literature surrounding nutrition in sepsis, with emphasis on lipids, and the potential therapeutic use of palmitate, butyrate and alpha-linolenic acid in modulating immune function, mitochondrial function and metabolism.

Methods: An *in vitro* model using human peripheral blood mononuclear cells exposed to lipopolysaccharide (LPS) or clinical strains of bacteria was used to determine the effects of palmitate (long-chain, saturated), butyrate (short-chain, saturated) and alpha-linolenic acid (ALA) (long-chain, unsaturated) on immune and mitochondrial function in sepsis via cytokine secretion, cell-specific flow cytometric analyses and mitochondrial respiration. A rat faecal peritonitis model was then utilised to investigate the impact of intravenous butyrate infusion on metabolism, immune function and mitochondrial function.

Results: Palmitate, butyrate and alpha-linolenic acid had pro-, anti-, and mixed inflammatory effects on cytokine secretion respectively. Butyrate, but not palmitate or alpha-linolenic acid, increased maximal mitochondrial respiration and spare respiratory capacity. Butyrate infusion *in vivo* stimulated fatty acid metabolism, but did not impact on immune function and may increase mitochondrial stress.

Conclusions: Palmitate and butyrate could potentially impact on sepsis pathology, but at different phases of sepsis. Beneficial *in vitro* effects of butyrate on immune and mitochondrial function could not be reproduced *in vivo*. Measuring plasma butyrate levels should be addressed in future studies.

Impact statement

Sepsis is a major clinical problem with high incidence and mortality. Current treatment is mostly based on a 'one-size-fits-all' strategy and the impact of nutrition is under-addressed. Besides being an important source of calories, nutrition also has a pharmacological impact. Fatty acids impact upon immune function, mitochondrial function and metabolism in different ways, and their individualised use may contribute to a personalised medicine approach depending on the patient's immune and bioenergetic status. In this thesis, I show that different fatty acids variably affect immune and mitochondrial function which may impact on septic patients depending on their characteristics and the specific phase of sepsis they are in. I also demonstrate in an *in vivo* rat model of sepsis that although 'patients' may look the same, their immune, mitochondrial and metabolic markers may differ markedly. This thesis therefore contributes to increased awareness on the need for a personalised medicine approach to sepsis and offers new potential therapies. My research also utilises new methodologies for cell-specific *in vitro* models of sepsis. Together, this thesis contributes to sepsis research methodology, and provides new insights in the way nutrition could be used to modulate disease processes.

Acknowledgements

I would like to thank my primary supervisor, Professor Mervyn Singer. His humour and passion for intensive care medicine has been very motivational and educational over the course of this PhD. Many thanks also to my secondary supervisor, Dr. Manu Shankar-Hari, for his meaningful input and guidance throughout.

I would like to thank Professor Thierry Roger, who gave me the opportunity to work in his lab and be part of his team at Lausanne University Hospital, Switzerland, for a 7-month secondment.

Thanks to all colleagues at the Singer lab, for the daily doses of scientific discussions, humour, lab runs and socials.

I would like to thank the Marie-Curie EU Horizon 2020 'European Sepsis Academy' Award for my funding. I am grateful to have been part of this project and be surrounded by students and professionals in the field.

Thanks to all my friends for your support, interest, fun (sports) activities and stayovers, and warm welcomes in London and Lausanne.

Special thanks to my family, mostly my parents, my sister and my brother who have encouraged me throughout and moved me to and within different countries.

Thank you, Luuk, for holding on to our long-distance relationship all the way through.

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AA   arachidonic acid

ADP   adenosine diphosphate

ALA   alpha-linolenic acid

ANT   adenine nucleotide translocase

APC   antigen presenting cell

ATP   adenosine diphosphate

AUC   area under the curve

BE   base excess

BSA   bovine albumin serum

BTLA   B and T lymphocyte attenuator

CD   cluster of differentiation

CLR   C-type lectin receptor

CO   cardiac output

COX   lipid-peroxidising enzyme cyclooxygenase

CPT   carnitine palmitoyltransferase

CTLA-4   cytotoxic T-lymphocyte-associate antigen 4

DAMP   damage-associated molecular pattern

DC   dendritic cell

DHA   docosahexaenoic acid

DMSO	dimethyl sulfoxide
DPA	docosapentaenoic acid
EC	*Escherichia coli*
ECAR	extracellular acidification rate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESPEN	European Society for Parenteral and Enteral Nutrition
EPA	eicosapentaenoic acid
ETC	electron transport chain
FABP	fatty acid binding protein
FADH$_2$	flavin adenine dinucleotide
FAO	fatty acid oxidation
FASN	fatty acid synthase
FBS	fetal bovine serum
FCCP	carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
FFAR	free fatty acid receptor
FFA	free fatty acid
GLUT	glucose transporter
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPR	G protein-coupled receptor
HDAC	histone deacetylase

HCl	hydrochloric acid
HBSS	Hank's balanced salt sodium
HDL	high-density lipoprotein
HIF	hypoxia-inducible factor
HLA	human leukocyte antigen
HPA axis	hypothalamic-pituitary-adrenal axis
HR	heart rate
HSL	hormone-sensitive lipase
i.p.	intraperitoneal
ICU	intensive care unit
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IV	intravenous
IVFE	intravenous fat emulsion
LCAT	lecithin-cholesterol acyltransferase
LCFA	long-chain fatty acid
LCT	long-chain triglyceride
LDL	low-density lipoprotein
LOX	15-lipoxygenase
LPL	lipoprotein lipase

LPS	lipopolysaccharide
LT	leukotriene
LXR	nuclear receptor liver X receptor
MAC	membrane attack complex
MCFA	medium-chain fatty acid
MCT	medium-chain triglyceride
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
mPTP	mitochondrial permeability transition pore
mTORC1	mTOR complex 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	nicotinamide adenine dinucleotide
NET	neutrophil extracellular trap
NF-κB	nuclear factor-κB
NH_4Cl	ammonium chloride
NK	natural killer
NLR	NOD-like receptor
NO	nitric oxide
NOD	nucleotide oligomerization domain
NRF-1	nuclear respiratory factor 1
OCR	oxygen consumption rate

ONOO⁻   peroxynitrite

OxPhos   oxidative phosphorylation

PAMP   pathogen-associated molecular pattern

PBMC   peripheral blood mononuclear cell

PBS   phosphate-buffered saline

PD-1   programmed cell death-1

PD-L   programmed death ligand

PGC-1α   peroxisome proliferator-activated receptor gamma coactivator 1α

PGN   peptidoglycan

PG   prostaglandin

PICS   persistent inflammation, immunosuppression and catabolism
        syndrome

PMA   phorbol 12-myristate 13-acetate

PPAR   peroxisome proliferator-activated receptor

PPP   pentose phosphate pathway

PRR   pattern recognition receptor

PTP   permeability transition pore

PUFA   polyunsaturated fatty acid

RER   respiratory exchange ratio

RCF   relative centrifugal field

RIG   retinoic acid inducible gene

ROS   reactive oxygen species

RQ   respiratory quotient

SA   *Staphylococcus aureus*

SAPE   streptavidin-PE

SCFA   short-chain fatty acid

SDS   sodium dodecyl sulphate

SREBP   sterol responsive element binding protein

STAT   signal transducer and activator of transcription

SV   stroke volume

TAG   triacylglycerol

TCA   tricarboxylic acid

TCR   T-cell receptor

Tfam   transcription factor A for the mitochondrion

Th   T helper

TLRs   Toll-like receptors

TMRM   tetramethylrhodamine methyl ester

TNF   tumor necrosis factor

Treg   regulatory T cell

UCP   uncoupling protein

VLDL   very-low density lipoprotein

VTI   velocity-time integral

Chapter 1   Introduction

Sepsis is a major clinical problem with high incidence and mortality. Current treatment is mostly based on a 'one-size-fits-all' strategy and the impact of nutrition is under-addressed. Besides being an important source of calories, nutrition also has a pharmacological impact. Fatty acids impact upon immune function, mitochondrial function and metabolism in different ways, and their individualised use may contribute to a personalised medicine approach depending on the patient's immune and bioenergetic status. This thesis describes the role of palmitate, butyrate and alpha-linolenic acid on immune and metabolic function in sepsis.

1.1   Sepsis definition

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Septic shock is a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone [1]. With 11 million sepsis-related deaths, sepsis represents 19.7% of all global deaths and represents a major health problem [2]. Sepsis is the predominant cause of mortality in the intensive care unit (ICU) [3]. However, older patients are far more likely to develop sepsis, are more likely to die, and older survivors are less likely to be discharged home [4]. The reported incidence of sepsis is increasing, particular as the population ages and more invasive procedures are performed [5, 6]. Survivors often have long-term psychological, physical, and cognitive disabilities with substantial healthcare and social implications [7, 8].

1.2   Immune function in health

The human body is exposed daily to many xenobiotic, potential pathogenic compounds. When a pathogen passes the first line of defence (i.e. skin, mucus membranes, etc.), the host's immune system provides protection. The immune system classically consists of an innate and adaptive immune system. Immune cells derive from pluripotent stem cells in the bone marrow, of which innate immune

cells derive from myeloid precursor and adaptive immune cells from lymphoid precursor. Most innate immune cells (neutrophils, basophil, eusinophils, monocytes) mature in bone marrow, whereas natural killer (NK) cells are from lymphoid lineage and mature in the thymus. Monocytes in the periphery can differentiate into dendritic cells (DCs) and macrophages. Lymphoid cells of the adaptive immune system mature in the bone marrow (B cells) or in the thymus (T cells) and reside and secondary lymphoid organs (e.g. lymph nodes and spleen) and circulate in the blood [9].

Cells of the innate immune system detect pathogen-associated molecular patterns (PAMPs) such as endotoxin and DNA via pattern recognition receptors (PRRs) [10]. PRRs can also detect damage-associated molecular patterns (DAMPs) released by injured host tissue. Several classes of PRR families have been identified. These include (i) transmembrane proteins such as C-type lectin receptors (CLRs) that recognise fungi, (ii) Toll-like receptors (TLRs) and cytoplasmic proteins such as retinoic acid inducible gene I-like (RIG-I like) receptors that recognise viral RNA and DNA, and (iii) nucleotide oligomerisation domain (NOD)-like receptors (NLRs) [10, 11]. NOD1 and NOD2 detect bacterial peptidoglycan (PGN) fragments [10] while TLRs recognise different molecular patterns of various microorganisms. TLRs are the best studied with 13 receptors reported in mice and humans, as shown in Table 1-1 [12].

Table 1-1: Pattern recognition receptors: subcellular localisation, and recognised ligands.

Receptor	Subcellular localisation	Ligand	Origin of ligand
TLR2	Cell surface	Lipoteichoic acid	Gram + bacteria
		Lipoprotein/lipopeptides	Various pathogens
		Hemagglutinin protein	Viruses (Measles Virus)
		Glycosyl-phosphatidylinositols	Parasites
TLR2/1	Cell surface	Triacyl lipopeptides	Gram - bacteria, mycobacteria
TLR2/6	Cell surface	Diacyl lipopeptides	Gram + bacteria, mycobacteria
		Zymosan	Fungi
TLR3	Cellular compartment	dsRNA	Viruses
TLR4	Cell surface	Lipopolysaccharide	Gram - bacteria
		Envelope proteins	Viruses
		Glycosyl-phosphatidylinositols	Parasites
TLR5	Cell surface	Flagellin	Bacteria
TLR7/8	Cellular compartment	ssRNA	Viruses
TLR9	Cellular compartment/ cell surface	CpG-containing DNA	Bacteria, viruses
TLR11	Cellular compartment	Uropathogenic bacteria component	Bacteria (*Escherichia coli*)
		Profilin	Parasites
TLR12	Cellular compartment	Profilin	Parasites
TLR13	Cellular compartment	bacterial ribosomal RNA sequence "CGGAAAGACC"	Bacteria, viruses
NOD1	Cell cytoplasm	Meso-diaminopimetic acid	PGN of Gram + and some Gram - bacteria
NOD2	Cell cytoplasm	Muramyl dipeptide	PGN of Gram + and some Gram -bacteria
RIG-1	Cell cytoplasm	5'-triphosphate-bearing RNAs	Viruses

Gram +, Gram-positive; Gram -, Gram-negative; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; PGN, peptidoglycan.

In addition to recognising structural features via PRRs, the innate immune system also recognises structural features through missing-self recognition discriminating self from non-self [13, 14]. Upon recognition, the innate immune system initiates production of a cascade of mainly pro-inflammatory mediators, but also anti-inflammatory [11] and tissue-repair responses [15]. These trigger multiple downstream processes that aim to defend the body against the pathogen [16, 17]. Innate immunity responds rapidly, is non-specific and recruits immune cells to the site of inflammation through production of cytokines and chemokines [18]. Translocation of nuclear factor-κB (NF-κB) into the nucleus and subsequent target gene activation (e.g. those encoding for cytokines) are crucial for induction of inflammation [17].

Innate immunity consists of cellular and non-cellular elements. Phagocytes (predominantly macrophages and neutrophils) are crucial components of innate immunity by engulfing and killing pathogens. Neutrophils are short-lived and contain granules and enzyme pathways that eliminate pathogens. Macrophages live longer and, like DCs, are antigen-presenting cells (APCs) developed from monocytes, capable of activating the adaptive immune response by presenting antigens on major histocompatibility complexes (MHCs) for recognition by T cells. NK cells help to mobilise APCs via secretion of interferon gamma (IFN-γ) and release of perforins and granzymes from NK-cell granules that induce apoptosis. Other innate immune cells are mast cells, basophils, eosinophils and innate lymphoid cells [18].

An important non-cellular element of innate immunity is the complement system: a biochemical cascade that opsonises pathogens, making pathogens susceptible to phagocytosis. Other non-cellular elements include acute-phase proteins, anti-microbial peptides, secretory IgA and circulatory IgM [15]. The innate immune system hence consists of physical, chemical and cellular elements [18].

The innate immune system provides crucial information to the adaptive immune system [15]. The interplay between innate and adaptive immunity is crucial when innate immunity is ineffective in eliminating the pathogen [18]. Classically, thought

innate immune responses were considered to have no immunologic memory. However, Netea *et al*. introduced the concept of trained innate immunity, describing an immunological memory of past insults for innate host defence [19].

The adaptive immune system is well known for its memory capacity, enabling the host to mount a more rapid and efficient immune response upon secondary exposure to the same antigen. Adaptive immunity is antigen-dependent and specific, however maximal response time after antigen exposure is longer compared to innate immunity (days vs. hours) [18].

Cells of the adaptive immune system include T cells and B cells. T cells derive from the bone marrow and mature in the thymus. T cells express unique antigen-binding receptors on their membrane, known as T cell receptors (TCRs). The MHC-antigen complex on APCs activates TCRs, enabling T cells to rapidly proliferate and differentiate and secrete cytokines which further control immune responses. T cells primarily differentiate into cytotoxic T cells (CD8+ cells) or T-helper (Th) cells (CD4+ cells). Cytotoxic T cells destroy infected cells and are important in eliminating tumour cells. T helper cells (Th1, Th2 and Th17 being most frequent) maximise immune responses and are potent cytokine secretors. Regulatory T cells (Tregs) are a subset of CD4+ cells that suppress immune responses, thereby controlling responses to self-antigens and resolving normal immune responses upon elimination of antigens. Tregs play an important role in immune tolerance [18]. T cells are further known to have memory function [20].

B cells arise from haematopoietic stem cells in the bone marrow where they mature. B cells can recognise antigens directly, independent of APCs. When activated by foreign antigens, B cells proliferate and differentiate into antibody-secreting plasma cells or memory B cells. T helper cells secrete cytokines that help B cells to proliferate and mature into antibody-producing plasma cells, with production of IgA, IgD, IgE, IgG and IgM antibodies. Secreted antibodies bind to antigens on the surface of pathogens and this is important for neutralisation, complement activation and opsonisation processes. Memory B cells are long-lived and respond quickly upon antigen re-exposure by producing antibodies [18].

In most cases, innate and adaptive immune responses are efficient in eliminating the pathogen via a protective and balanced response, followed by a return to homeostasis. However, in some infections, pathogens survive and multiply despite an activated immune system, and the host response can become unbalanced and harmful.

1.3   Immune function in sepsis

1.3.1   Pro-inflammatory responses in sepsis

Sepsis is characterised by an initial excessive pro-inflammatory response to a pathogen. Pro-inflammatory cytokines implicated in sepsis pathogenesis include tumor necrosis factor (TNF, also known as TNFα), interleukin-1β (IL-1β), IL-12 and IL-18 [21]. Although an augmented anti-inflammatory response is generated simultaneously to control the flames of unbridled inflammation [22], early deaths in sepsis are generally the result of cardiovascular collapse and multi-organ dysfunction, likely driven by exaggerated inflammatory reactions [17].

Activation of the complement system, coagulation pathways and endothelial activation enable passage of immune cells into the infected tissue and local microvascular obstruction to prevent systemic spread of the pathogen. In sepsis, these responses become dysregulated and play a significant role in the early excessive pro-inflammatory phase of sepsis.

1.3.1.1   Complement activation

Upon activation by either bacterial polysaccharides or IgM and IgG antibodies, the complement system releases complement peptides, with C3a and C5a having potent pro-inflammatory effects. These effects include recruitment and activation of leukocytes, endothelial cells and platelets [23]. Platelets augment immune cell recruitment and inflammation, facilitate the formation of vaso-occlusive thrombi in capillary vascular beds, and can have direct cell toxic effects. Excessive platelet activation likely contributes to organ injury [24]. The complement system is also important in the formation of a membrane attack complex (MAC) that results in cell

lysis [25]. Complement activation is crucial in protective immunity, but its uncontrolled activation can cause damage to tissues and organ failure [17].

1.3.1.2 Coagulation activation

Coagulation activation also plays an important role in inflammation in sepsis, of which the tissue factor pathway is the main driver [26]. Thrombin, factor Xa, and tissue factor VIIa (involved in the tissue factor pathway) are recognised by protease-activated receptors on activated endothelial cells, neutrophils and monocytes, leading to an increased synthesis of pro-inflammatory mediators (e.g. IL-6, IL-8) and adhesion molecules [17]. Leukocytes, particularly neutrophils, are recruited by adhesion molecules expressed on the vasculature. Neutrophils release lytic enzymes, reactive oxygen species (ROS) and nitrogen intermediates, contributing to microcirculatory and organ failure in sepsis [27].

Excessive activation of the coagulation system in sepsis is associated with disseminated intravascular coagulation. This is clinically associated with microvascular (and, occasionally, macrovascular) thrombosis and haemorrhage, the latter resulting from rapid consumption of coagulation factors [27].

1.3.1.3 Endothelial activation

The exaggerated inflammatory response in sepsis activates the endothelium, resulting in release of multiple pro- or anti-inflammatory mediators, including eicosanoids and nitric oxide, and also compromises endothelial barrier integrity. There is increased rolling and adherence of leukocytes to the endothelial surface, with transmigration of these cells through the blood vessels to the tissues beyond. While an appropriate protective response in areas of active infection where bacteria are multiplying, in sepsis this can occur indiscriminately into non-infected tissues. Similarly, increased barrier incompetency causes leakage of intravascular proteins and plasma into the extravascular space; while benefitting infected areas with ingress of complement, immunoglobulins and other protective molecules, in sepsis there is often widespread tissue oedema and reduced microvascular perfusion [17].

1.3.2 Immunosuppression in sepsis

After the initial immune activation, an immunosuppressive state dominates in sepsis (Figure 1-2). This can be argued as a protective response [17] that dampens the early pro-inflammatory response by a robust depletion of both innate and adaptive immune cells, such as NK cells, CD4+ T cells, CD8+ T cells, B cells and DCs, through apoptosis [28]. A striking loss of immune cells is reported in autopsy studies in patients who died of sepsis [29]. The authors considered increased lymphocyte apoptosis to be causative, but also altered bone marrow functionality contributes. Bone marrow function in sepsis is suppressed and there is increased release of myeloid-derived suppressor cells (MDSCs) [30, 31] and immature neutrophils [32]. Bone marrow suppression in sepsis is further characterised by inhibition of early T cell lineage progenitor differentiation [33], which is associated with loss of thymocytes due to apoptosis. Thus, in addition to circulating cells, systemic immunosuppression affects both primary and secondary lymphoid organs after sepsis. There are corresponding decreases in antibody production, macrophage activation, and antigen presentation [29]. Neutrophils show delayed apoptosis [17]. Neutrophils are initially mobilised and activated. However, subsequently released neutrophils have reduced bacteriocidal functionality and decreased cytokine production, and increased release of the immunosuppressive cytokine, IL-10 [34]. Tregs and MDSCs however expand in number while APCs are reprogrammed. APCs, including monocytes, macrophages and dendritic cells, have reduced expression of human leukocyte antigen (HLA)-DR (an MHC class II molecule) and therefore function less well in recognising antigens; they also have a diminished capacity to produce pro-inflammatory cytokines [17].

Boomer *et al.* reported a profound depletion of T, B and dendritic cells in both murine models of sepsis [35, 36] and septic patients [28, 37-39]. Post-mortem analyses of spleens and lymph nodes taken from patients who died of sepsis showed a significant loss of CD4+ and CD8+ T cells [28]. This loss was thought to be caused by the rapid release of pro-inflammatory cytokines, especially TNF [40]. The release of complement factor C5a induces apoptosis by binding to the C5a receptor on thymocytes [41]. T cells additionally show increased expression of the immune

checkpoints, programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associate antigen-4 (CTLA-4) [28], contributing to increased cell death and cellular unresponsiveness [42]. CTLA-4 and PD-1 both inhibit immune responses, though CTLA-4 regulates T-cell proliferation early in the immune response and primarily in lymph nodes, whereas PD-1 suppresses T cells later on and primarily in peripheral tissues [43]. Signalling through PD-1 inhibits T-cell proliferation, induces IL-10 secretion and inhibits cytotoxicity of CD8+ T cells [44, 45]. Similarly, B and T lymphocyte attenuator (BTLA) and programmed death ligand (PD-L) are upregulated in B cells, and monocytes [28, 37]. There is decreased expression of CD28 on T cells taken from septic patients compared to healthy controls [28]. Without co-stimulation through CD28/B7 when the T cell receptor engages antigen, T cells become functionally unresponsive, or die through neglect [46]. Anergic patients often have decreased Th1 functionality but no increase in Th2 (anti-inflammatory) cytokine production [47]. Failure of T cells to proliferate or secrete cytokines is associated with an increased mortality risk [47]. Stimulated cytokine secretion in sepsis is generally <10% of that measured in non-septic cells [28]. There is also increased expression of selected inhibitory receptors and ligands, expansion of suppressor cell populations but extensive depletion of splenic CD4+, CD8+ and HLA-DR cells.

Altered functionality of monocyte, macrophage, neutrophil and lymphocyte populations as described above can affect the balance between pro- and anti-inflammatory responses [48]. Surviving CD4+ and CD8+ T cells shift from a pro-inflammatory Th1 towards an anti-inflammatory Th2 phenotype [34]. Patients with burns, trauma and sepsis often have decreased levels of Th1 cytokines (e.g. TNF, IFN-γ, IL-2), but increased levels of Th2 cytokines (e.g. IL-4 and IL-10) [49, 50].

Table 1-2: Effects on immune cell function in sepsis-induced immunosuppression

Cell type	Changes in sepsis-induced immunosuppression
NK cells	Depletion
T cells	Depletion of CD4+ and CD8+ T cells, increased PD-1 and CTLA-4 expression, decreased CD28 expression, decreased cytokine production, Treg expansion
B cells	Depletion, increased BTLA and PD-L expression, decreased antibody production
Monocytes and dendritic cells	Depletion, reduced HLA-DR expression
Neutrophils	Delayed apoptosis, reduced bactericidal functionality, decreased cytokine production, increased IL-10

With the above-mentioned changes summarised in Table 1-2, the critically ill patient is at increased risk of secondary infection such as pneumonia. This risk is enhanced as patients are unnaturally immobile, mechanically ventilated, and have external surfaces breached by catheters and drains for many days, if not weeks. This is compounded by the near-universal use of sedative agents and antibiotics, and the frequent use of vasoactive catecholamines, all of which are immunosuppressive to variable degrees [51]. Septic patients may develop 'chronic critical illness', also referred to as the 'persistent inflammation, immunosuppression and catabolism syndrome' (PICS) [52, 53].

It was initially believed that the excessive inflammatory response in sepsis was the major cause of mortality. However, most deaths from sepsis now occur later and often during a prolonged immunosuppressed state that can last for weeks, if not longer [54]. Immune suppression may persist for many months, even after other organs have seemingly recovered their full functionality [22].

There may be a role for anti-inflammatory strategies (e.g. corticosteroids) early in sepsis in those patients exhibiting an excessive pro-inflammatory response. Later

on, immunosuppressed patients may benefit from activation of monocyte/lymphocyte numbers and functionality using activators such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, PD-1 inhibitors, or IL-7 [55]. Multiple trials have targeted early phase inflammation; unfortunately, all have shown either no benefit or worse outcomes [56]. It is now recognised that both excessive inflammation and immune suppression, together with a failure to return to homeostasis, are key survival factors [46].

1.4 Mitochondrial function in health

Cells need adenosine triphosphate (ATP) to maintain their functionality. In most cells, a relatively small amount of ATP is produced in the cytosol independent of oxygen via glycolysis, with most being produced by mitochondria through oxidative phosphorylation (OxPhos). These organelles use most of the body's oxygen consumption to generate ATP. Glucose, amino acids and fatty acids are used as substrates for the tricarboxylic acid (TCA) cycle. Amino acids may enter directly or via acetyl-CoA, whereas glucose and fatty acids are first converted to acetyl-CoA via glycolysis and β-oxidation, respectively. Acetyl-CoA enters the TCA cycle and donates electrons to the electron transport chain (ETC) via NADH and $FADH_2$. The ETC consists of enzyme complex I-IV, transporters ubiquinone and cytochrome c, and ATP synthase (complex V). As electrons are moved down the chain, protons are pumped across the inner mitochondrial membrane creating a proton gradient. This proton-motive force is used by ATP synthase to generate ATP through phosphorylation of adenosine diphosphate (ADP). Adenine nucleotide translocase (ANT) assists ATP/ADP exchange across the mitochondrial membrane and transports ATP out of the mitochondria [57, 58]. Electron transfer terminates at complex IV, where oxygen is reduced to water. Premature or incomplete reduction of oxygen will produce superoxide radical, 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 oxygen consumption [59]. Mitochondrial antioxidants such as manganese superoxide dismutase and glutathione protect mitochondria from damage induced by ROS. ROS however also play an important role in signalling, immunomodulation, vascular tone maintenance and oxygen sensing [57].

Some of the energy present in the proton gradient dissipates as heat by re-entry of H^+ ions into the mitochondrial matrix without conversion to ATP [60]. This incomplete coupling is called proton leak or mitochondrial uncoupling. Proton leak is closely linked to mitochondrial membrane potential: a higher membrane potential increases proton leak, while raised proton leak rate increases oxygen consumption and decreases membrane potential [61]. Decreasing membrane potential represents a protective mechanism by decreasing ROS production and protecting mitochondria from oxidative damage [62]. ROS, in turn, activate uncoupling, further supporting the protective mechanism of uncoupling [63].

Proton leak can be both basal and inducible. Basal proton leak is cell-type specific, depends on the inner mitochondrial membrane composition and can utilise up to 30-50% of basal metabolic rate [61]. Mechanisms are not fully understood but ANTs are likely involved [64]. Four members exist in humans (ANT-1 to -4). Besides their role in ATP handling, ANTs have uncoupling potency that can account for up to 50% of basal mitochondrial membrane proton conductance [65].

Inducible proton leak or uncoupled respiration is mediated via ANTs and uncoupling proteins (UCPs) (Figure 1-1) and can be activated by superoxide, peroxidation products and fatty acids [61]. Five different UCPs are found in human (UCP-1 to -5), expressed in different tissues. UCP-1 is predominantly expressed in brown adipose tissue (BAT) where it accounts for approximately 10% of mitochondrial protein content. It plays a thermogenic role through increased proton leak, with up to 20% of energy dissipated as heat [60].

Although strong evidence exists for UCP-1 and its uncoupling potency [66, 67], the role of other UCPs remains uncertain [68-70]. UCP-2 and UCP-3 are mainly responsible for inducible proton leak outside BAT. UCP-2 is expressed in many tissues with high levels found in white adipose tissue, skeletal muscle, spleen, pancreatic β-cells [71] and cells of the innate immune system [72, 73]. UCP-3 mRNA is predominantly found in skeletal muscle, heart and to a lesser extent, adipose tissue [71]. Unlike UCP-1, UCP-2 and UCP-3 are only activated under specific circumstances (e.g. high FFAs levels in sepsis [74, 75]) and with much lower

expression [58]. Their uncoupling potency is also lower compared to UCP-1, but their mild uncoupling may play a protective role by reducing oxidative stress as described above.

In 1979, Hunter and Haworth discovered that inner mitochondrial membranes harbour an unselective pore that is triggered by calcium and is regulated by nucleotides [76, 77]. The structure of this mitochondrial permeability transition pore (mPTP) is still a matter of debate [78]. mPTP opening enables free passage of small molecules (<1.5 kDa) into the mitochondria, including protons [76, 77]. Opening of the mPTP is triggered by mitochondrial calcium overload (especially in combination with oxidative stress), elevated phosphate concentrations, adenine nucleotide (ATP and ADP) depletion [76], and dissipation of the mitochondrial membrane potential [79]. Opening of the mPTP results in uncoupling of oxidative phosphorylation and ATP depletion. ANT was first thought to play a central role, but is now identified to have a regulatory role [78, 79]. Free fatty acids also induce mPTP opening [80], as discussed later. The inhibitory effect of adenine nucleotides is thought to be via the ANT, as the potency of nucleotides as inhibitors of mPTP opening is correlated with their ability to act as substrates for ANT [81, 82]. Halestrap and others proposed a model where CyP-D binds to ANT which undergoes a conformational change into a pore when triggered by calcium [83-86]. ANT was shown to be accountable for almost half of the basal proton conductance across the inner mitochondrial membrane, even when blocked [65]. This suggests a direct regulatory role for ANT on mitochondrial proton gradients, and indirectly for other ions. The role of ANT on mPTP modulation hence appears indirect; effects on other proteins are reviewed elsewhere [78].
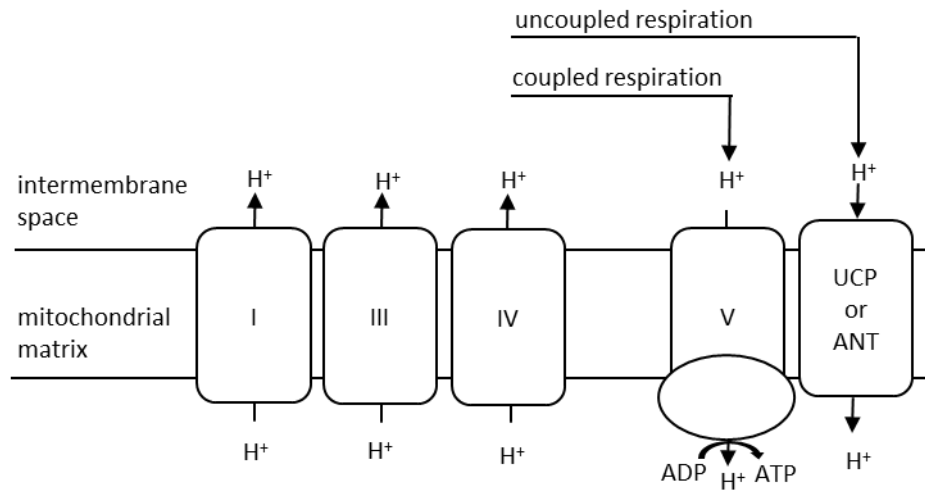
Figure 1-1 Mitochondrial coupled and uncoupled respiration.

UCP: uncoupling protein; ANT: adenosine nucleotide translocase.

Mitochondria have many physiological functions other than ATP and ROS production, including regulating immune function [87], involvement in apoptosis [88], and anabolism and catabolism of metabolites.

1.5   Mitochondrial function in sepsis

While immune dysfunction increases the patient's risk of secondary infection, this does not explain why organs fail. Notably, minimal cell death is found in patients who die from multiple organ failure [38]. Notably, tissue oxygen tensions are preserved and survivors have a relatively rapid recovery of organ function, even in those organs that are poorly regenerative [89-91]. Mitochondrial dysfunction can plausibly explain this functional rather than structural abnormality underlying organ failure [3].

Mitochondrial function may be affected by various processes in sepsis. Tissue hypoxia, and hence insufficient oxygen delivery at a mitochondrial level to drive oxidative phosphorylation, may occur through impaired perfusion early in sepsis as a result of fluid losses, myocardial depression, microcirculatory redistribution of blood flow and loss of vascular tone [92, 93]. Septic patients produce excessive amounts of nitric oxide (NO) that, together with its metabolites, can directly inhibit several respiratory enzymes and thus affect oxidative phosphorylation. Disruption

of the respiratory chain increases the formation of superoxide (O_2^-) that reacts with NO to generate peroxynitrite ($ONOO^-$). $ONOO^-$ is a powerful oxidant, capable of denaturing mitochondrial proteins, cleaving DNA, and causing prolonged/irreversible inhibition of the respiratory chain and a decrease in ATP synthesis [3]. This oxidant stress overwhelms intra-mitochondrial defence mechanisms [94].

My host lab previously showed that the degree of NO production is associated with decreased activity of mitochondrial complex I and lower ATP levels in skeletal muscle of patients with septic shock [95], as well as in muscle and liver in a long-term rodent model of faecal peritonitis [96]. These studies reported an association between increased NO and decreased glutathione, an important antioxidant for detoxifying peroxynitrite and hydrogen peroxide, and maintaining protein thiol groups in a reduced state. Decreased glutathione was also related to illness severity [95, 96].

NO can however induce mitochondrial recovery (biogenesis) in various cell types via the key regulator, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), [97]. Upregulation of mitochondrial biogenesis markers such as PGC-1α, Tfam (transcription factor A for the mitochondrion) and NRF-1 (nuclear respiratory factor 1) was associated with recovery of metabolic activity, organ function and clinical improvement in a long-term mouse model of *S. aureus* peritonitis [98]. Muscle biopsies of septic survivors revealed an increased mRNA content of PGC-1α [99]. Eventual survivors had higher levels of skeletal muscle ATP and the mitochondrial antioxidant, manganese superoxide dismutase. Increased mitochondrial size suggestive of swelling was associated with worse outcomes [99]. A study of post-mortem kidney and heart samples showed hydropic mitochondria while immunohistochemistry showed few apoptotic cells [100].

Prolonged elevation of stress hormone levels such as catecholamines and cortisol, and decreased levels of anabolic, thyroid and sex hormones may also negatively impact upon mitochondrial function [101]. Sepsis further triggers decreased transcription of genes encoding mitochondrial proteins [99], and increases

mitochondrial uncoupling [102], dissipating the proton gradient and diverting oxygen consumption away from ATP production towards generation of heat.

The net result of all the above processes is a decrease in ATP production. Non-mitochondrial ATP production through glycolysis would still be possible, but the ATP yield per mole of glucose is much lower. Bioenergetic failure may possibly represent an adaptive, protective response, allowing continued integrity of the cell with the potential to recover when the insult has passed and inflammation has reduced [3]. Singer *et al.* proposed a 'hypometabolic' theory where cells protect themselves from ATP depletion during sepsis by lowering cellular metabolic demands to prevent the fall in ATP levels that would trigger cell death pathways. This has similarities with the metabolic shutdown seen in hibernating or estivating animals in response to a prolonged, harsh environment [103].

Another, more clinically relevant example, of purinergic signalling is observed in the heart [104]. In response to low ATP concentration caused by coronary artery occlusion or ischaemia, vasodilatation of coronary arteries and downregulation of energy expenditure occurs in order to maintain intracellular ATP levels. Although exact mechanisms are not fully understood, several studies demonstrated cardioprotective effects of adenosine (a breakdown product of ATP by ecto-enzymes) by reducing ischaemic damage and enhancing cardiac function during severe hypothermia [105], and suppressing contractility of right ventricular trabeculae and preserving electrical stability [106]. Increased hydrolyses of ATP to AMP has also shown myocardial protection against cardiac ischaemia-reperfusion injury [107], together demonstrating another example of purinergic signalling, as well as a protective value of reduced ATP levels.

1.6   Metabolic phases in sepsis

Like immune and mitochondrial function, different metabolic phases are described in sepsis. Although timing and exact course varies per patient, sepsis is generally characterised by an initial hypermetabolic phase where the body actively fights the infection; a subsequent hypometabolic phase, akin to hibernation, where many of

the normal metabolic activities are shut down; and a late hypermetabolic phase that helps drive anabolism and recovery.

The phases described below describe a textbook picture of metabolic phases in sepsis. In clinical practise however, patients do not present these phases so clearly. This is likely due to the high person-to-person variability, and one-size-fits-all approach does not hold true. For example, timings can vary significantly. A study dating from 2000 followed sequential metabolic changes in critically ill patients with severe sepsis or major trauma. They showed that over a 3-week period, both patient groups lost 13% of their total body protein. Also their resting energy expenditure was progressively increased over the first week and was still elevated three weeks after onset of illness. This prolonged hypermetabolism was not reflected by plasma levels of proinflammatory cytokines, which fell rapidly over the first week of illness [108]. The timing of hypermetabolism differenced substantially from a study on infants after gastrointestinal surgery, where it was shown that resting energy expenditure peaked at 4 hours after surgery but returned to baseline within 12 hours [109].

That age may influence metabolism in critical illness is shown in a study on energy expenditure in critically ill children. Here, authors measured resting energy expenditure within 24 hours of admission and noted that critically ill children do not become hypermetabolic. Only a small number of children had elevated metabolism at some point during their admission, but this was not consistent or predictable. Although some studies report elevated energy metabolism in critically children [110, 111], stronger evidence demonstrates hypometabolism [111, 112, 113, 114].

The impact of patients' heterogeneity and hypermetabolism was also shown in a single-center, prospective, six-month observational study in China [115], where the authors linked early hypermetabolism (defined as high resting energy expenditure) to poor outcome as estimated by 28-day mortality rate and prolonged hospital stay. Authors noted that most ICU patients presented hypermetabolism during the first 5 days after admission as measured by indirect calorimetry. CRP was also elevated during this phase, especially in the hypermetabolic group rather than the

normometabolic group. Interestingly, despite similar critically ill condition, the hypermetabolic group had a significant higher proportion of men than women, a finding also found elsewhere [115].

Next to individual factors such as gender, age, and body mass index, patients' metabolism is further influenced by demographics, comorbidities, medication and treatment. Given these many confounders, indirect calorimetry measuring oxygen consumption and carbon dioxide production is generally considered the gold standard for measuring resting energy expenditure, rather than predictive equations (such as Schofield and Fleisch equations) based on individual factors. This was confirmed in two separate studies on critically children with severe sepsis, where authors found that predictive equations were inaccurate compared with indirect calorimetry [116, 117].

Given the enormous variability in metabolism described above, the following section on metabolic phases provides an oversimplified picture of looking at metabolism in the critically ill.

1.6.1 Initial hypermetabolic phase

The host initially demands a greater energy supply to meet the increased metabolic requirements necessary to fight infection. This acute stress response to injury is driven by sympathetic stimulation, activation of the hypothalamic-pituitary-adrenal (HPA) axis (increased production/release of cortisol, aldosterone) and release of other stress hormones such as glucagon and vasopressin [101, 118]. The increased release of cortisol, glucagon and catecholamines (e.g. adrenaline, noradrenaline) induce catabolism with breakdown of protein, especially from muscle, an increased reliance on fat and a reduced dependence on carbohydrate metabolism with accompanying insulin resistance.

Mitochondrial respiration is elevated and ATP production enhanced during this early phase [119, 120]. Some of the increase in total body oxygen consumption may also result from diversion of the proton motive force across the inner mitochondrial membrane away from oxidative phosphorylation towards uncoupled respiration

and production of heat [121]. This may be looked at as a dysfunction, however, this increase in uncoupling (which can also be caused by fatty acids) lowers mitochondrial membrane potential and may offer protection by reducing ROS production, as explained in more detail later in section 1.10.

1.6.2 Hypometabolic phase

Most patients recover promptly from an infectious insult, either with or without antibiotics. The patient may suffer of unwellness related to the systemic inflammatory response with release of cytokines and other mediators that influence drivers of metabolism including behaviour (e.g. taking to bed), loss of appetite, changes in regional blood flow, altered hormone levels and substrate utilisation [103]. A small proportion of infected patients however progress to organ dysfunction, i.e. sepsis. This can be relatively mild or much more severe with varying degrees of physiological and biochemical abnormality, usually affecting multiple organ systems [16, 103]. This often requires admission to critical care for increased monitoring and more sophisticated organ support. The initial hypermetabolic phase generally lasts from days to weeks, followed by a hypometabolic phase [119, 120] (Figure 1-2). Timing and course can however differ as described above.

In the hypometabolic phase, organ dysfunction can progress further, or fail to recover, and this may last from days to months. Most patients, at least in high-income countries, now die during this phase as modern medical technology often supports the patient through their initial, acutely unwell phase [54]. The hypometabolic phase extends across multiple systems. These range, for instance, from the kidney [122] with persisting low urine output and uraemia, to the gut [123] with prolonged ileus and diarrhoea, and to the immune system with immunosuppression and increased susceptibility to secondary infections [48]. Muscle wasting is commonplace; this is, in part, related to infection-related myopathy and neuropathy, but also to disuse atrophy and autocannibalism with utilisation of muscle substrate (protein and fat) by other organs [124]. Total oxygen consumption falls during this period [125].

1.6.3 Hypermetabolic recovery phase

Recovery from critical illness requires considerable energy to meet the demands necessary for renewed functioning of organs and independence from life support machines. Energy is needed to restore intact endothelial and epithelial barriers, to grow epithelial and other cells (for example, intestinal villi), to restore muscle bulk and strength, to rid the body of excess salt and water accumulated over the period of critical illness, and to mobilise/rehabilitate.

A corresponding increase in oxygen and substrate utilisation occurs during this recovery phase, with a significant rise in whole body oxygen consumption [126, 127]. Feeding should ideally be increased to meet these increased metabolic demands.

Figure 1-2 provides a simplified overview of immune and metabolic phases in sepsis. Of note, both pro- and anti-inflammatory responses are activated at the same time in the early phase. Furthermore, patient's immune and metabolic phases are not as clear as depicted in Figure 1-2 and exact course and timing can differ.
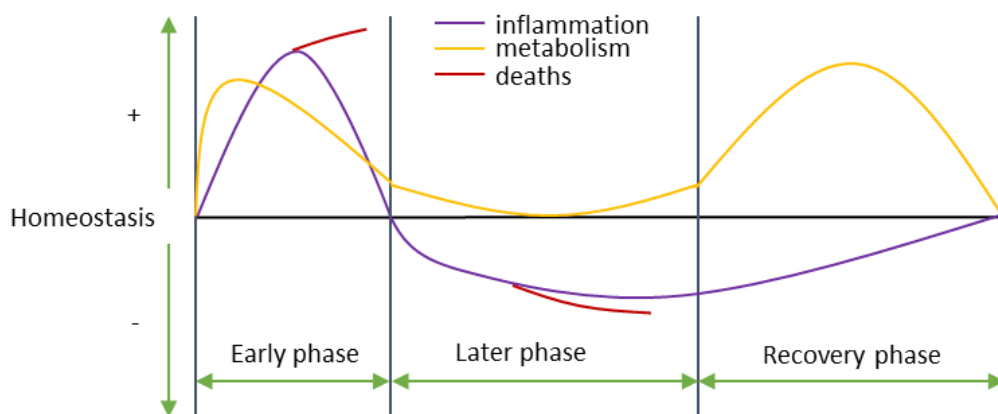
Figure 1-2: Immune and metabolic phases in sepsis.

1.7   Substrate metabolism in sepsis

Carbohydrate, protein and fat metabolism undergo changes in sepsis. These are summarised in Figure 1-4 and described in more detail below.

1.7.1   Carbohydrate metabolism in sepsis

As part of the acute stress response, glucose levels rise in the blood [128]. Glucose stores are rapidly utilised to meet the increased energy demands of the initial hypermetabolic response. This allows rapid availability of substrate to hypermetabolic organs such as brain and exercising muscle. Thereafter, glucose is provided by glycogen breakdown (glycogenolysis) from muscle and liver, but these stores are depleted within 12-16 hours while gluconeogenesis is much reduced during sepsis [129]. Glucose provision then comes from hepatic and renal generation of glucose from non-carbohydrate sources such as glucogenic amino acids, triglycerides, glycerol and lactate (gluconeogenesis), as well as exogenous administration.

The early rise in glucose levels is driven by increases in counter-regulatory hormones (cortisol, glucagon, catecholamines) that antagonise the actions of insulin. These also prevent activity of insulin-dependent cell glucose transporters (GLUT) such as GLUT4 which accounts for most glucose transport into muscle and adipose cells [130].

Increased cytokine release in early sepsis is associated with insulin resistance, reducing use of glucose as a mitochondrial substrate. TNF induces hyperglycaemia and insulin resistance [131]. IL-1β induces a hypermetabolic state and works synergistically with IL-6 [132]. Insulin resistance at the cellular level is further induced via stimulation of hepatic gluconeogenesis and release of FFAs from adipose tissue [101]. The resulting hyperglycaemia can impair functionality of the innate immune system, increasing the risk of secondary infection [133].

1.7.1.1   Lactate metabolism in sepsis

In response to a decrease in oxidative phosphorylation, anaerobic respiration (glycolysis) is upregulated to partially compensate for the fall in ATP production

[134]. This is however relatively short-lived and less efficient. For each mole of glucose, a net total of two moles of ATP are generated through glycolysis. By comparison, the Krebs cycle generates two moles and the electron transport chain approximately 26-30 moles of ATP. In addition to the two ATP molecules, two pyruvate molecules are also produced from each molecule of glucose. Pyruvate is converted by pyruvate dehydrogenase to acetyl-CoA which then enters the TCA cycle. However, pyruvate dehydrogenase activity is reduced in septic patients [135], hence limiting the use of glucose as a mitochondrial substrate. Pyruvate not taken up into mitochondria is converted by lactate dehydrogenase into two molecules of lactate. This, together with additional mechanisms such as enhanced adrenaline-driven sodium pump activity by skeletal muscle and impaired lactate clearance by the liver, results in an increase in plasma lactate levels [132]. The lactate can be recycled back to pyruvate and used as an energy substrate by other organs.

The blood lactate level is a biomarker widely used by clinicians caring for patients with sepsis. In sepsis, increased glycolysis is associated with increased local and systemic lactate levels, although impaired lactate clearance also contributes [136, 137]. However, lactate is a nonspecific marker, and also rises with tissue hypoxia, excessive activation of glycolysis driven by catecholamines even under aerobic conditions, or mitochondrial dysfunction unrelated to oxygen deficiency [138]. Failure for lactate to normalise during resuscitation is associated with a poor prognosis [132].

Although lactate is generally considered as a waste product of glucose metabolism, it becomes increasingly clear lactate plays a pivotal role in diverse biological processes, including immune cell polarisation, differentiation and effector functions as reviewed elsewhere [139, 140]. In inflammatory disease microenvironment, lactate triggers a series of intracellular signals that promoting chronic inflammatory processes [141] [139, 142, 143]. Lactate further showed to upregulate de novo fatty acid synthesis in CD4+ T cells, leading to enhanced production of IL-17 and reduced cell motility [141], linking metabolism to immune function as described in more detail later in section 1.9.

Cellular uptake of lactate is mediated via various H+ and Na+/K+ - coupled electroneutral transporters expressed on epithelial cells, cancer cells, fibroblasts and immune cells, emphasising its widespread effector functions [139]. The Na+/K+ - coupled electroneutral transporters are also important in the uptake of other small molecules as short-chain fatty acids (SCFAs) as discussed later.

1.7.2 Protein metabolism in sepsis

Accelerated proteolysis occurs during sepsis [144]. Amino acids (particularly, alanine and glutamine) are released primarily from muscle for gluconeogenesis, for production of acute phase reactants by the liver, and to serve as an energy substrate for the liver and rapidly dividing cells of the intestinal mucosa and the immune system. Essential amino acids such as leucine, isoleucine and valine are also used as energy sources [144]. The consequence of protein breakdown and no compensation of synthesis leads to a net negative nitrogen balance [131]. This leads to skeletal muscle wasting, deconditioning, and a prolonged recovery time [132]. Critically ill patients have profound loss of muscle mass, with 17% mean loss of femoral mass by day 10, and greater losses in the more severely ill [145]. Apart from muscle, gut, lungs and kidneys are also significantly affected by increased protein breakdown [144].

1.7.3 Lipid metabolism in sepsis

Fat is the largest store of energy within the body; up to 25% of body weight can be adipose tissue [146]. Metabolic fuel is stored as triacylglycerol (TAG). After eating fat, TAG molecules are assembled within enterocytes and attached to chylomicrons that reside in the intestine [147]. Very-low density lipoproteins (VLDL) produced in the liver carry almost all triglycerides from the liver via the bloodstream to adipose tissue for storage, or to muscle for utilisation as a fuel substrate. For tissue utilisation, the TAG component of chylomicrons and VLDL is hydrolysed by lipoprotein lipase (LPL), releasing fatty acids that can be stored in adipose tissue or muscle [148]. Plasma insulin levels regulate LPL activity in both adipose tissue and muscle [148]. In muscle, VLDL-TAG is hydrolysed in muscle capillaries by LPL,

yielding more fatty acids. These fatty acids can be oxidised directly [149] or stored in the muscle TAG pool [150] (Figure 1-3).
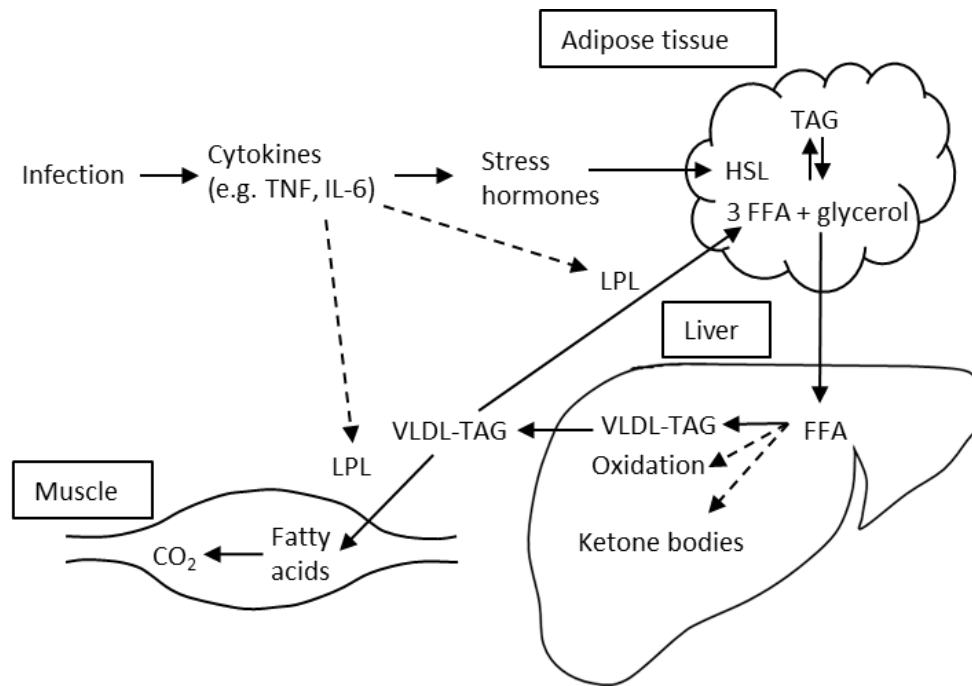
Figure 1-3: Effect of sepsis on lipid metabolism.

LPL: lipoprotein lipase; HSL: hormone-sensitive lipase; TAG: triacylglycerol; FFA: free fatty acid; VLDL: very low-density lipoprotein; CO_2: carbon dioxide. Solid arrow: stimulation. Dashed arrow: inhibition.

Fatty acid oxidation (FAO) is enhanced in sepsis, as reflected by a lower respiratory exchange rate (RER) [151]. The RER is the ratio of carbon dioxide (CO_2) produced to oxygen (O_2) utilised (VCO_2/VO_2). At rest, RER equals the respiratory quotient (RQ). The ratio gives an indication of what substrate is used in energy metabolism with RER values of 1.0 for carbohydrate, 0.83 for protein and 0.70 for fat [152]. In my host lab's long-term rat model, the RER of septic animals fell within hours to 0.75-0.80 whereas in control animals it remained between 0.90-1.0 (unpublished data). This implies a change in metabolic substrate in septic animals away from carbohydrate towards a mixture of fat and protein utilisation [152]. This shift was also seen in a caecal ligation and puncture sepsis model [153].

Alterations in lipid metabolism in sepsis may be caused by LPL inhibition, upregulated hepatic triglyceride production caused by hyperglycaemia and hyperinsulinaemia, disruption of the synthesis-utilisation balance, and interactions with cytokines, endotoxin and hormones [148, 154].

LPL activity was reduced in muscle and adipose tissue biopsies taken from patients with sepsis [155]. Increased adipose tissue lipolysis provides the fatty acids needed for VLDL synthesis in the liver, thus raising triglyceride levels even in the presence of decreased lipid absorption [156, 157].

Endotoxin (lipopolysaccharide, part of the outer membrane of Gram-negative bacterial cell walls) can suppress LPL activity through TNF production, thereby lowering VLDL clearance by peripheral tissues [148]. In response to endotoxin, TNF is quickly released and can increase lipolysis both directly [158], and indirectly through other cytokines [159]. Other cytokines further down the inflammatory cascade have similar indirect effects [148]. Animal studies have shown that both TNF and IL-6 can increase hepatic lipogenesis [160, 161]. In humans, administration of TNF and IFN-γ induced hypertriglyceridaemia in a similar manner [162, 163].

Lipid metabolism plays a crucial role in macrophage polarisation towards an inflammatory phenotype. Indeed, fatty acid synthase deletion was shown to reduce macrophage recruitment in a model of diet-induced inflammation in mice [164].

Activation of the autonomic nervous system with increased secretion of catecholamines stimulates catabolism and lipolysis and augments release of FFAs in the critically ill patient [148, 165]. Hormone-sensitive lipase (HSL) is inhibited by insulin and stimulated by catecholamines, cortisol and growth hormone [166]. HSL hydrolyses the triacylglycerol stored in adipose tissue and produces FFAs and glycerol that are then released into the circulation [167]. Catecholamines also increase adipose tissue blood flow, further contributing to increased lipolysis and raised FFA concentrations [168, 169]. Hence, extra substrates are available for oxidation to meet the increased energy demands of the stressed patient.

Septic patients have decreased plasma levels of the cholesterol-carrying lipoproteins, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) [148]. Plasma cholesterol is typically low due to decreased hepatic synthesis and increased consumption [170]. The decrease in LDL and HDL suggests derangements of lipid metabolism in the intravascular compartment and may be due to impaired lecithin-cholesterol acyltransferase (LCAT) activity [171]. LCAT is the key enzyme for production of cholesteryl esters in plasma promotes HDL formation [172] and is suppressed in human experimental endotoxaemia and clinical sepsis [173].

Both HDL and LDL play an important role in protection against sepsis. HDL clears bacterial toxins via reverse cholesterol transport, modulates innate cellular immunity, prevents inflammatory cytokine release, and transports cholesterol to the adrenal glands for steroid synthesis [174-178]. LDL can also clear bacterial toxins and provides substrates for steroid synthesis [174]. HDL and LDL however become dysregulated in sepsis as acute and chronic inflammation oxidises lipoprotein particles [179-181]. Oxidised HDL, but also oxidised LDL, makes these lipoproteins dysfunctional and pro-inflammatory [182]. Cholesterol feeding on its own elevates circulating markers of inflammation (e.g. C-reactive protein, serum amyloid A) in lean subject [183]. Obese humans often have systemic inflammation, reflected by increased circulating C-reactive protein and cytokines [184-186]. Expansion of adipose tissue is related to secretion of inflammatory mediators (e.g. cytokines) by resident and infiltrating macrophages, inducing low-grade systemic inflammation [187]. Inflammatory pathways are also activated in liver [188, 189] and the vasculature [190] and metabolically active tissues by lipids [191].

The inflammatory response of fatty acids is dependent on their structural properties, as discussed in more detail below and summarised in Table 1-3.

1.8   Fatty acids and immune function

Depending on their hydrocarbon chain lengths, degree of unsaturation (number of double bonds between carbon atoms), number, position and orientation of their double bonds, lipids have differential effects on immune function and metabolism [170]. Based on chain length, fatty acids are divided into long-chain fatty acids

(LCFAs) with a carbon length of 16-22 carbon atoms, medium-chain fatty acids (MCFAs) with 8-14 carbon chain lengths and short-chain fatty acids (SCFAs) consisting of 2-8 carbon chain lengths.

Fatty acids are the main constituents of cell membranes and are involved in hormone synthesis, cell signalling and lipid-soluble vitamin transport [192]. Alterations in membrane fatty acid composition may alter protein activities and cell functions [170]. Membrane phospholipid composition of immune cells can be modified by exogenous fatty acids. Most of the modulatory effects of fatty acids on inflammation can be attributed to fatty acid metabolites such as prostaglandins, resolvins, leukotoxins, endocannabinoids, diacylglycerols and ceramides [193].

The molecular organisation of lipid rafts can also be modulated by alterations in membrane phospholipid composition. Lipid rafts are membrane domains with specific structural compositions [194, 195]. They play a major role in cell signalling and immune responses, including T-cell activation, signal transduction and protein and lipid trafficking [170]. Different lipid raft compositions and organisations alter B cell functionality [196], T cell signalling and immune synapse formation [197].

Fatty acids are also natural ligands of the transcription factor family of peroxisome proliferator-activated receptors (PPARs). PPARs are members of the superfamily of nuclear hormone receptors, including receptors for steroid hormones and fat-soluble vitamins A and D [198]. All three known PPARs can bind fatty acids, preferably poly-unsaturated fatty acids (PUFAs) [199-203]. Fatty acid-derived compounds, or those with structural resemblances, can also activate PPARs [204-211]. PPARs play a crucial role in gene expression, particularly genes involved in lipid metabolism (including their β-oxidation), oxidative phosphorylation and differentiation of immune cells toward an anti-inflammatory phenotype [212].

PUFAs are further involved in inhibition of sterol-responsive element binding proteins (SREBPs)-1 and -2 [213]. SREBP-1 induces expression of genes involved in lipid synthesis in liver and adipose tissue [214]. This, in turn, decreases the ability of SREBP-1 to activate transcription of SREBP-responsive lipogenic genes in the liver [215, 216]. SREBP-1 levels are inversely associated with mRNA levels of the

uncoupling proteins UCP-2 and UCP-3 in adipose tissue [217, 218] and skeletal muscle [219, 220], respectively. The possible relevance of UCPs in sepsis is discussed later on.

Downregulation of SREBP-1 mRNA by PUFAs may be mediated by antagonising the activity of nuclear receptor liver X receptors (LXRs) which are potent inducers of SREBP-1 gene transcription [221, 222]. Both LXRs and SREBPs are involved in regulation of macrophage activation. LXRs antagonise pro-inflammatory transcription factors, such as NF-κB [223, 224] and, via activation of anti-inflammatory genes, inhibit inflammatory responses [225, 226]. SREBP-1 however promotes an acute inflammatory response via production of IL-1β and formation of the inflammasome [227, 228]. Additionally, the LXR pathway is negatively regulated by TLR4 [229].

Saturated fatty acids like palmitate show mostly pro-inflammatory effects. They activate TLR4 which then activates NF-κB. Most of the biological activity of lipopolysaccharide (LPS) is mediated via its lipid A moiety, which structurally resembles saturated fatty acids [213]. Saturated fatty acids may further activate TLR4 by promoting its recruitment to lipid rafts via a mechanism involving ROS [230].

Unsaturated fatty acids do not activate TLR4. n-3 PUFAs like alpha-linolenic acid are mostly anti-inflammatory [193]. Alpha-linolenic acid biosynthesis leads to the production of multiple n-3 fatty acids via desaturations, elongations and oxidation [231]. These n-3 fatty acids include stearidonic acid, eicosatetraenoic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). n-3 PUFAs compete with arachidonic acid (AA, n-6-PUFA) for enzymatic metabolism, thereby decreasing the production of pro-inflammatory metabolites from AA via 15-lipoxygenase (LOX) and the lipid-peroxidising enzyme cyclooxygenase (COX) pathway. These pro-inflammatory mediators include prostaglandins (PGs) and leukotrienes (LTs) [170]. Other anti-inflammatory properties of n-3 fatty acids include inhibition of NF-κB transcription with a decrease in inflammatory gene expression and activation of anti-inflammatory

transcription factors [232, 233]. n-3 PUFAs also reduce pro-inflammatory cytokine and ROS production and increase the synthesis of anti-inflammatory cytokines. By contrast, n-6 PUFAs show pro-inflammatory properties [57, 58].

SCFAs such as butyrate are generally anti-inflammatory and are involved in chemotaxis, apoptosis, proliferation, differentiation and gene expression [234]. Signalling pathways include activation of G protein-coupled receptors (GPRs, some are also known as free fatty acid receptors (FFARs)), inhibition of histone deacetylases (HDACs), stimulation of histone acetyltransferase activity, and stabilisation of hypoxia-inducible factor (HIF) [235-238], thereby influencing innate and adaptive immune responses. [234].

Some GPRs on cell membranes can be activated by SCFAs. In particular, GPR41 and GPR43 are highly expressed in the colon, where high concentrations of SCFAs exist through bacterial fermentation [239]. GPR41 is expressed on numerous immune cells while GPR43 is involved in the resolution of inflammatory responses [240]. MCFAs and LCFAs can activate other GPRs, such as GPR-40, -84 and -120 [241].

Table 1-3 Fatty acids and immune function.

Fatty acid	Saturation	Inflammation	Signalling
SCFA		Anti-inflammatory	- GPR - HDAC inhibition - HIF stabilisation - Histone acetyltransferase stimulation - NF-κB inhibition
LCFA	Saturated	Pro-inflammatory	- Altering membrane composition - TLR4 activation -NF-κB activation - Lipid raft modulation
	Unsaturated: Omega-3	Anti-inflammatory	- PPAR - SREBP inhibition - LXR activation -Compete with pro-inflammatory metabolites for enzymatic metabolism - Nf-κB inhibition
	Unsaturated: Omega-6	Pro-inflammatory	- PPAR - production of pro-inflammatory metabolites (e.g. prostaglandins and leukotrienes)

SCFA: short-chain fatty acid; LCFA: long-chain fatty acid; GPR: G protein-coupled receptor; HDAC: histone deacetylase; HIF: hypoxia-inducible factor; NF-κB: nuclear factor κB; TLR: toll-like receptor; PPAR: peroxisome proliferator-activated receptor; SREBP: sterol-responsive element binding protein; LXR: liver X receptors
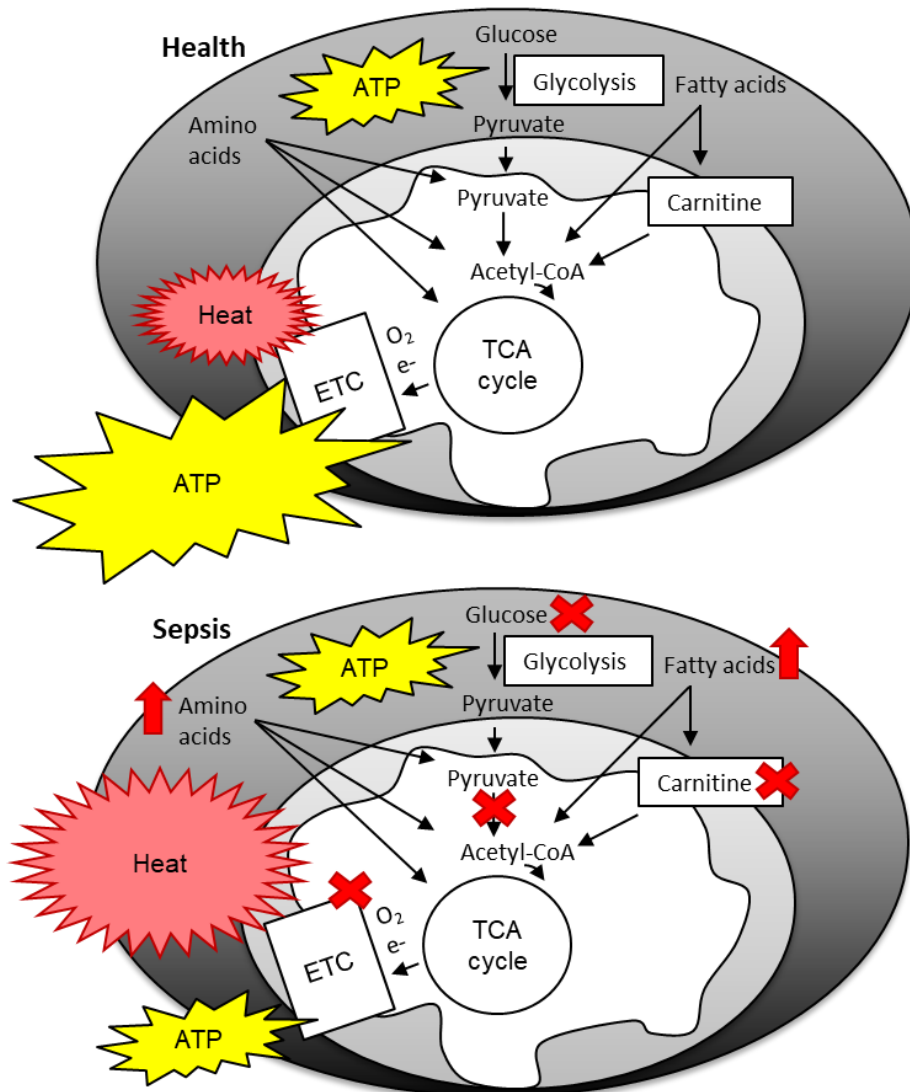
Figure 1-4: Mitochondrial function in health and sepsis.

ETC: electron transport chain; TCA tricarboxylic acid

1.8.1.1 Mitochondrial uptake of fatty acids

Mitochondrial oxidation of fatty acids involves import of fatty acid into the mitochondrial matrix via carnitine palmitoyltransferase-1 (CPT1) and -2 (CPT2). This requires carnitine as a co-factor. LCFAs completely depend on carnitine for mitochondrial uptake [242]. MCFAs and SCFAs are carnitine transport-independent [63, 64]. Carnitine may be depleted in sepsis due to reduced renal reabsorption with elevated urinary carnitine loss, muscle wasting or decreased tissue uptake [243, 244]. These processes limit transport of LCFAs into mitochondria (Figure 1-4).

MCFAs and SCFAs are less dependent on transport mechanisms; whereas MCFAs and SCFAs can be transported in the blood as free FAs bound to albumin, LCFAs need to be coupled to acyl-CoA and packed into chylomicrons for transport from the gut to the blood through the lymphatic system [245]. Fatty acid-binding proteins (FABPs) and fatty acid translocase (FAT/CD36) are responsible for cytosolic trafficking of LCFAs and transfer across the plasma membrane, respectively. These are downregulated in sepsis and endotoxaemia [246], hence limiting the availability of LCFAs for mitochondrial oxidation. SCFA reach the cell cytoplasm via passive diffusion across the plasma membrane (in non-ionised form), or through transporters (e.g. Slc16a1 and Slc5a8) [234]. Both SCFA and MCFA depend to a lesser extent on fatty acid binding proteins [247].

The multiple metabolic pathways involved make the effects of fatty acids *in situ* complex. These effects are dependent on their physical properties, mechanisms of cellular uptake and intracellular transfer, competition with intracellular stores and the bioactivities of fatty acids and their metabolic products [248].

1.9   Bioenergetics in immune cells

Nutrient and substrate restrictions in sepsis may contribute to reduced ATP production. This triggers AMPK activation to suppress anabolism yet promote catabolic pathways [249]. Oxygen availability may be limited in damaged tissue sites and this local deficiency may also affect metabolism [250]. Nutrient restrictions can have profound effects on immune responses. Immune cells can rapidly ramp up glycolytic activity when activated. They can also switch metabolism based on substrate availability, and this may help to maintain immune system functionality under stress [251]. Immune cells can use both glucose and free fatty acids to generate ATP, and to mount [252-254], maintain [255] or resolve inflammation [256]. Pro-inflammatory and effector immune cells generally use aerobic glycolysis for immediate ATP demand, whereas anti-inflammatory, naïve and tolerant cells depend more on fatty acid oxidation [253, 257, 258].

Other than erythrocytes, all cell types contain mitochondria though numbers vary markedly. For example, cardiomyocytes are packed with mitochondria, occupying at

least 30% of their cell volume in humans [259]. Their main role in muscle is to produce sufficient ATP to enable ongoing contractility and relaxation, and to respond promptly to increases in whole body activity [260]. Depending on the immune cell type, there is a greater or lesser dependence on mitochondria-generated ATP to fuel metabolic processes. Glucose and fatty acids are the most important substrates used by these cells [261]. Generally speaking, naïve and tolerant cells rely on fatty acid oxidation for ATP generation [256, 257, 262], whereas effector cells mainly depend on aerobic glycolysis [253, 258].

1.9.1 Neutrophils

Neutrophils are the most abundant circulating leukocytes and have essential roles in innate immunity [263]. The neutrophil contains few mitochondria per cell [264] and a negligible oxygen consumption rate [265]. These cells predominantly utilise glucose through glycolysis. To a minor extent, the pentose phosphate pathway (PPP, a branch of glycolysis generating NADPH and pentose sugars) also supports neutrophil bioenergetics [251]. Dissipation of the mitochondrial membrane potential, as found upon LPS exposure, also reduces the neutrophil respiratory burst, thereby reducing ROS production [266].

1.9.2 Macrophages

On encountering pathogens, monocytes are rapidly activated and migrate to the affected area where they differentiate into pro-inflammatory M1 or anti-inflammatory M2 macrophages [267]. Activated M1 macrophages switch metabolically from oxidative phosphorylation to glycolysis [268]. This provides substrates for biosynthetic programs, for ATP production and to maintain mitochondrial membrane potential [249]. M1 macrophages have high rates of glucose and glutamine uptake and lactate production [269, 270]. Inhibiting oxidative phosphorylation increases ROS production, exerting antimicrobial activities [249].

Upon resolution of inflammation, macrophages transform into the M2 phenotype with more oxidative phosphorylation [271]. M2 macrophages have higher rates of fatty acid oxidation and mitochondrial biogenesis [272]. Whereas HIF-1α is a

53

primary regulator of glycolytic metabolism in M1 macrophages [266], signal transducer and activator of transcription (STAT6 promotes expression of genes involved in fatty acid oxidation and OxPhos via PGC-1β. Expression of PPARs is also upregulated [273].

1.9.3 Dendritic cells

At rest, dendritic cells oxidise glucose through OxPhos, producing relatively little lactate. DCs switch to glycolysis within minutes upon pro-inflammatory activation [270] and glycolysis remains elevated with loss of OxPhos over 18 hours. This has considerable impact in regulating DC-induced T cell responses [274].

Upon stimulation with TLR agonists, DCs become dependent on Warburg metabolism for survival [270]. The Warburg effect describes how cells metabolise glucose into lactate even in the presence of sufficient oxygen to support OxPhos, so called 'aerobic glycolysis'. In cells activated for over 12 hours, glucose no longer enters the TCA cycle and mitochondrial oxygen consumption ceases [270, 275]. The Warburg effect has been observed for cancer cells [276]. PI3K and Akt play a key role in glycolytic metabolism [277, 278] and, accordingly, are essential in activated DCs [270].

Activation of DCs leads to expression of inducible nitric oxide synthase (iNOS) that inhibits mitochondrial respiration via excess production of nitric oxide [275]. A similar effect on mitochondrial respiration due to high NO production has been described in macrophages [279].

1.9.4 B cells

Not much literature exists on B cell bioenergetics. Activation of the B cell receptor is regulated by ROS [280, 281]. Upon activation, B cells increase glucose and glutamine metabolism during clonal expansion comparable to T cells [282, 283].

1.9.5 T cells

T cells also undergo a metabolic switch once activated. Whereas naïve T cells rely on glucose oxidation via OxPhos [266] and fatty acid oxidation [284], enhanced

glycolysis via PPP and glutaminolysis support rapid growth, proliferation and acquisition of specialised effector functions when stimulated via antigen or cytokine receptor-dependent mechanisms [285]. T cells are metabolically similar to tumour cells, as both use Warburg metabolism when proliferating [251]. Most innate immune cells also engage Warburg metabolism when activated, but do not proliferate [251].

Upon CD28 stimulation, PI3K and Akt increase GLUT1 abundance on the T cell membrane surface [285]. Along with enhancing glucose uptake by activated T cells, this also induces a switch from OxPhos to glycolytic metabolism via mTOR [285, 286]. Activation of mTOR complex 1 (mTORC1) in mouse embryo fibroblasts stimulated glycolysis and OxPhos, plus *de novo* synthesis of lipids [287]. Induction of *de novo* fatty acid synthesis is essential for activation-induced proliferation and differentiation of effector T cells, especially CD4+ T cell differentiation [288]. Fatty acid catabolism via β-oxidation is important for CD8+ T cell memory and for differentiation of CD4+ regulatory T cells [288].

AMPK plays an important role in the induction of fatty acid oxidation in CD8+ T cells [289]. AMPK may affect fatty acid transport by increasing cellular fatty acid uptake for its oxidation [290]. Additionally, AMPK is a negative regulator of mTORC1 function [289, 291].

Activated T helper cells (i.e. Th1, Th2 and Th17) use glycolysis to support their effector functions [292], whereas Tregs predominantly use OxPhos and mitochondrial fatty acid oxidation for development and survival [293]. In contrast to effector T cells, memory T cells maintain a greater mitochondrial mass [294]; however, they do not utilise aerobic glycolysis, but predominantly use fatty acids to fuel OxPhos [295, 296].

1.10 Fatty acids and uncoupling

Depending on chain length, degree of saturation, and concentration, fatty acids have different effects on mitochondrial respiration [297]. Fatty acids are excellent respiratory substrates for most cell types, feeding electrons into the respiratory

chain. Fatty acids may also interact with mitochondrial membranes and alter their permeability [298]. Furthermore, fatty acids differently impact uncoupling.

1.10.1 Fatty acids as protonophores

FFA can act as protonophores and uncouple oxidative phosphorylation. LCFAs act as protonophores (an ionophore molecule able to translocate protons [58]) due to their structural composition: their negative charge is distributed over the molecule and they have a weakly ionised carboxylic group. Their hydrocarbon chain is of appropriate length without other binding atoms limiting mobility and hydrophobicity. LCFAs thus fit the structural prerequisites for proton-conducting mechanisms [299]. The uncoupling potency of fatty acids increases as chain length and unsaturation increase [297, 300, 301]. LCFAs increase basal respiration and uncoupling, and can induce proton leak and swelling of mitochondria via opening of the permeability transition pore [297]. They can also decrease mitochondrial membrane potential, thereby reducing ROS production [297, 300, 301].

In contrast to LCFAs, MCFAs and SCFAs have minor effects on mitochondrial swelling [302]. The uncoupling effect of fatty acids is generally lower as chain length decreases [301]. Shorter carbon chain lengths further reduce induction of proton permeability [303, 304]. MCFAs and SCFAs thus do not act as protonophores, probably due to their lower lipophilicity [305]. MCFAs and SCFAs only penetrate phospholipid membranes in a non-ionic form [306]. LCFAs are more lipophilic as their negative charge is more distributed over the molecule.

1.10.2 Other uncoupling mechanisms

Apart from acting as protonophores, fatty acids can impact on uncoupling by upregulating UCPs. UCP-1 is tightly regulated by purine nucleotides and free fatty acids, resulting in inactivation and activation, respectively [307]. UCP-1 is activated by fatty acids via mechanisms extensively reviewed elsewhere [308]. UCP-1 plays a thermogenic role through increased proton leak, with up to 20% of energy dissipated as heat [60]. Unlike UCP-1, UCP-2 and UCP-3 are only activated under specific circumstances (e.g. high FFAs levels in sepsis [74, 75]) and with much lower

expression [58]. UCP-2 and UCP-3 may also be less potent in uncoupling than UCP-1 [68-70].

UCP-2 may be involved in modulating ROS production and lipid handling, and exerts a central role in cellular metabolism [309]. UCP-3 may play a regulatory role in mitochondrial fatty acid oxidation and preventing ROS-induced oxidative damage [310]. Both UCP-2 and UCP-3 may be involved in glucose sensing [60, 68].

Besides upregulating UCPs, fatty acids activate ANT which plays a role in uncoupling [61] and mPTP formation [78], as described earlier. Although exact mechanisms are not yet fully understood, ANT plays a role in transporting FA anions across the inner mitochondrial membrane and hence mediates FA-induced uncoupling [311, 312]. This FA transport may involve cyclic movement of undissociated fatty acids, with the release of protons into the alkaline matrix space and subsequent efflux of fatty acid anions mediated by ANT [300, 312]. Other mitochondrial anion carriers (e.g. mitochondrial glutamate/aspartate, dicarboxylate, monocarboxylate and phosphate carriers) are also involved in FFA cycling across the inner mitochondrial membrane, but to a lower extent [298].

Fatty acids hence affect mitochondrial energy uncoupling by increasing proton conductance of the inner mitochondrial membrane which dissipates the proton gradient [299] and opens the mPTP [313]. Fatty acids further interact with mitochondrial membranes and may alter their permeability [298]. Opening of the mPTP may cause respiratory inhibition (and ultimately cell death) via the release of pyridine nucleotides from the matrix [314, 315], outer membrane rupture and cytochrome c release caused by swelling [316, 317]. Depolarisation and cell death caused by fatty acids are mostly due to mPTP opening rather than a direct effect on energy coupling, and effects tend to increase as unsaturation increases [297]. However, fatty acids may trigger other cell death mechanisms [298].

1.10.3 Uncoupling proteins and immune function

Mitochondria play a central role as cellular sensors that relay metabolic stress to the programming of the innate inflammatory response. This can either be via

stimulating or inhibiting inflammasome activation [318-321]. Fatty acid-induced mitochondrial uncoupling mediates inflammasome responses. Inflammasomes are families of innate receptors of the immune system that sense microbial infections and provoke metabolic inflammation at multiple levels [318]. In immune cells they form a molecular platform for caspase-1-dependent maturation and secretion of proinflammatory cytokines such as IL-1β and IL-18 [322]. Inflammasome activation is involved in insulin resistance, perturbed lipid metabolism and systemic inflammation [318]. The NLRP3 inflammasome recognises molecular patterns associated with host-derived metabolites. Lipids [323], hyperglycaemia [324] and amyloid proteins [325] can activate the NLRP3 inflammasome, triggering IL-1β secretion [318].

Metabolic derangements and mitochondrial dysfunction are associated with inflammasome activation and the pathogenesis of sepsis. During sepsis, UCP-2 regulates NLRP3-mediated caspase-1 activation in macrophages through upregulation of fatty acid synthase (FASN)-dependent lipid synthesis and activation of AKT and downstream p38MAPK [309].

1.11 Relevance of fatty acids in sepsis

Fatty acids may be beneficial in sepsis for their use as a substrate while glucose metabolism is impaired, and for their uncoupling potency and immunomodulatory effects.

Under normal physiological conditions, the uncoupling effect of fatty acids is negligible [300]. However, uncoupling may become apparent and influence cellular metabolism under physiological situations such as fasting, intense exercise [326, 327], cold stress, and pathological states such as diabetes [328], myocardial [329] and brain [300] ischaemia.

Mitochondrial uncoupling is often viewed as dysfunctional, but may offer multiple protective roles. Uncoupling is involved in autophagy activation (in particular mitophagy) [330], lipid disposal [331], ROS regulation by lowering membrane potential, reducing protein secretion by decreasing ATP content [332], calcium

homeostasis (as mitochondrial membrane potential is essential for Ca^{2+} to enter the mitochondria [333]), cell signalling (by reducing the ATP/AMP ratio and hence activating AMPK), and cell death [334]. The heat produced as a result of uncoupling may help to fight infection by direct bacteriocidal effects [335], by modulating immune cell activity [336], and through induction of heat shock proteins [337]. By lowering mitochondrial membrane potential and thus reducing ROS production, tissue damage in sepsis may be ameliorated.

In addition to their effects on uncoupling, fatty acids further impact immune function. These effects depend on their chemical structure and may be effective in modulating either the initial excessive pro-inflammatory or the later immunosuppressed phases of sepsis.

1.12 Hypothesis

Modulating fat metabolism in sepsis by supplying fatty acids will impact upon cellular bioenergetics and immune function, thereby affecting organ function, and thus survival. Depending on the type of fatty acid and the timing of intervention relative to the septic process, this may be beneficial or detrimental.

1.13 Aims and objectives

To investigate this hypothesis, I will utilise cell lines, primary cell isolates and cultures, and *in vivo* models. Specifically, I will:

1) study the impact of various fatty acids on immune function *in vitro*.
2) study the impact of various fatty acids on mitochondrial function *in vitro*.
3) characterise a long-term, fluid-resuscitated rat model of faecal peritonitis.
4) examine the impact of fatty acid infusion on metabolism, immune function and mitochondrial function in this *in vivo* model.

Chapter 2   Methodology

2.1   Isolation of peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were used to assess the effect of fatty acids on immune function. PBMCs include monocytes, T, B and NK cells, but not erythrocytes and platelets as they have no nuclei, nor granulocytes (neutrophils, basophils, and eosinophils) as these contain multi-lobed nuclei.

To obtain PBMCs, venous blood from healthy volunteers was obtained by phlebotomy and diluted 1:3 with PBS. For experiments requiring a lot of cells, buffy coat (Interregional Blood Transfusion SRC Ltd., Lausanne, Switzerland) from healthy blood donors were used and diluted 1:2 with PBS. Diluted blood was carefully layered on Ficoll-paque (GE Healthcare, Uppsala, Sweden) and centrifuged for 30 minutes at 400g without brake at room temperature. This separates the blood into plasma, PBMC, granulocyte and red blood cell layers (Figure 2-1). The PBMC layer was transferred into a clean tube and washed twice with PBS, after which the cells were ready to use. For freezing purposes, cells were diluted in FBS (ThermoFisher Scientific, Waltham, Mass, USA) + 10% DMSO (Thermo Fisher Scientific) and slowly frozen down to -80°C using controlled cell freezing boxes.
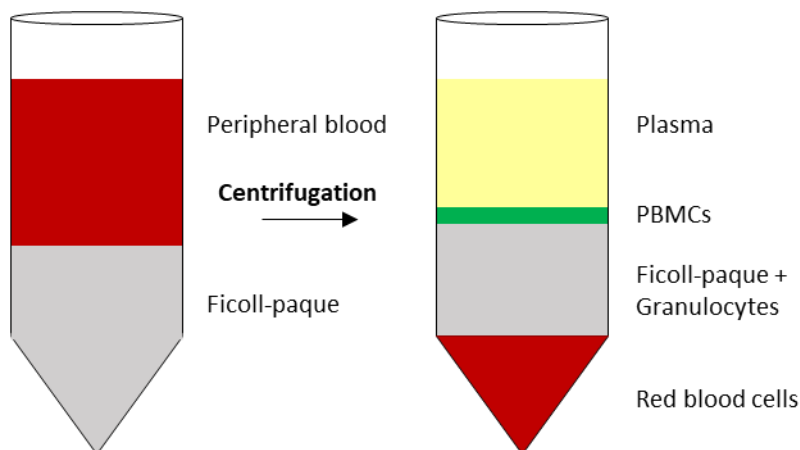
Figure 2-1: Peripheral blood mononuclear cell isolation using Ficoll-paque.

2.2   Fatty acid preparation

Prior to stock preparation, 0.1M NaOH and distilled water were prewarmed using a 70°C water bath. Palmitate stock solution was prepared by adding palmitate powder (Sigma-Aldrich, St. Louis, Missouri, USA) to prewarmed 0.1M NaOH. Butyrate stock solution was prepared by dissolving butyrate powder (Sigma-Aldrich) into prewarmed distilled water. Alpha-linolenic acid (ALA) in ethanol (Cayman Chemical, Ann Arbor, Michigan, USA) was supplied as a stock solution and could be used directly. All stocks were kept at -20°C until use.

2.3   Fatty acid dose-finding study

To test toxicity of various fatty acid concentrations, freshly isolated PBMCs from five healthy donors were cultured in RPMI 1640 GlutaMAX medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all ThermoFisher Scientific, further referred to as culture medium) in a round bottom 96-well plate at 37°C in 5% CO_2 at a density of 1×10^6 cells/well. PBMCs were incubated in triplicate for 4 hours and overnight with (i) LPS (100 ng/ml, Invivogen, San Diego, California, USA), (ii) clinically derived and heat-killed *Staphylococcus aureus* (*S. aureus*, SA) or (iii) *Escherichia coli* O18 (*E. coli*, EC) (PBMC:bacterium ratio 5:1) in the presence of palmitate (0.1, 0.4 and 2.2 mM), butyrate (0.1, 0.6 and 1.8 mM) or alpha-linolenic acid (ALA) (0.02, 0.1 and 0.3 mM). Fatty acid concentrations were in the physiological range (and lower for palmitate) [338, 339] or 3-fold elevated (butyrate, ALA) as previously measured in our septic rat model (Dr W Khaliq, unpublished data).

At the end of incubation, plates were centrifuged and supernatant carefully collected and stored at -20°C for cytokine analyses (section 2.9).

2.3.1   MTT assay

The MTT assay was used as a measure of cell viability. Metabolically active, hence viable, cells reduce the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) via NAD(P)H-dependent mitochondrial reductase to formazan, which has a purple colour.

61

MTT solution was prepared by dissolving MTT dye in IMDM (ThermoFisher Scientific, 1 mg MTT per ml IMDM). At the end of incubation, supernatant was collected as described before and cells then incubated for 2 hours at 37°C, 5% CO_2 with 100 µl MTT solution per well. MTT solution was removed and 100 µl lysing solution (a mixture of isopropanol, SDS 20% and HCl 5N) per well was added to dissolve the cells. Plates were covered and incubated overnight on a plate shaker. Absorbance was subsequently measured at 570 nm.

Five replicates per group were performed. For data analyses, fatty acid treated groups were compared to the control group (being stimulus without fatty acid) for each stimulus using the non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's correction for multiple comparisons.

2.4   Whole blood stimulation

As mentioned above, PBMCs include monocytes, T, B and NK cells, but, as opposed to human blood, no erythrocytes, platelets nor granulocytes (neutrophils, basophils, and eosinophils). Whole blood stimulation was performed to study the impact of fatty acids on immune function in whole blood.

Five healthy volunteers were requested to fast overnight to minimise blood fatty acid concentration. Venous blood was taken via phlebotomy and diluted five-fold in culture medium. Diluted blood was stimulated in triplicate with *E. coli* (10^7 bacteria/ml), *S. aureus* (10^8 bacteria/ml) and LPS (100 ng/ml) and treated overnight with palmitate (0.1 and 0.4 mM), butyrate (0.6 and 1.8 mM) and alpha-linolenic acid (0.1 and 0.3 mM).

At the end of incubation, plates were centrifuged and supernatant carefully collected and stored at -20°C for cytokine analyses, as described in section 2.9.

For data analyses, fatty acid treated groups were compared to the control group for each stimulus using the non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's correction for multiple comparisons.

2.4.1 Cytokine measurements by Luminex assay

ProcartaPlex Multiplex Immunoassay (ThermoFisher Scientific) was performed according to the manufacturer's protocol for measurement of IL-1β, IL-1RA and IL-6 using a Luminex system (Luminex Corporation, Austin, Texas, USA). In short, 25 μl magnetic beads were added to each well and placed on a hand-held magnetic plate washer for 2 minutes. Wells were washed with the provided wash buffer. Supernatant triplicates were pooled and 25 μl was then added to the each well. The plate was covered with a black lid and incubated for 30 minutes on a plate shaker at room temperature. The plate was then incubated overnight at 4°C.

The next day, the plate with black lid was incubated for 30 minutes on a plate shaker at room temperature. The plate was then washed twice using a hand-held magnetic plate washer. 12.5 μl detection antibody mixture was added to each well. The plate was incubated for 30 minutes at room temperature as before. It was then washed twice and incubated for another 30 minutes as before, with 25 μl Streptavidin-PE (SAPE) solution added. The plate was washed twice again and incubated with 65 μl reading buffer for 30 minutes as before prior to loading on to the Luminex.

Three biological replicates were performed. For statistical analyses, fatty acid treated groups were compared to the control group for each stimulus using the non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's correction for multiple comparisons.

2.4.2 Cytokine measurements by enzyme-linked immunosorbent assays (ELISA)

TNF and IL-10 secretion were analysed via enzyme-linked immunosorbent assays (ELISA, BD Biosciences) according to the manufacturer's protocol as described in section 2.9.

2.5 Flow cytometry

Flow cytometry was used to study the effects of palmitate, butyrate and alpha-linolenic acid on CD4+ helper T cells, B cells, dendritic cells and monocytes. Flow

cytometry is a tool developed in the late 1960s for phenotyping and sorting individual cells It offers an invaluable approach for understanding, monitoring and combating immune-related diseases [340, 341]. Suspensions of living cells are stained with specific, fluorescently labelled antibodies (fluorophore with marker of interest) and these stained cells are then loaded onto a flow cytometer. Each cell passes through one or more beams of focused light. This focused light is usually a laser that excites a single wavelength, ranging from ultraviolet to far red. Information on particle size and complexity is obtained by forward and side scatter, respectively. Emitted fluorescent light is detected by photomultiplier tubes, while specificity of detection is controlled by optical filters. Optical filters block certain wavelengths whilst transmitting others. Pulses are generated by detectors, reflecting the passage of particles through the laser beam. These pulses are mapped and can be used to compare fluorescent intensities and cell populations. When multiple fluorophores are used, these might be detected on multiple detectors. A mathematical method called 'compensation' can be applied to address possible spectral overlap between fluorophores in multiple detectors. The software calculates spillover values and will compensate the data to avoid false double positive populations. For data analyses, gates and regions are placed around cell populations with common characteristics. These characteristics are usually forward scatter, side scatter and markers of interest. [342]. A simplified overview of the technique is given in Figure 2-2.
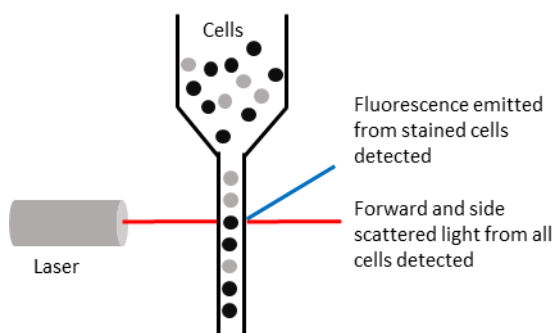
Figure 2-2: Simplified overview of flow cytometry.

Antibody panels (Table 2-1) were carefully chosen and include potential new therapeutic targets. The gating strategy is shown in Figure 2-3.

CD4+ T helper cells are characterised as $CD3^+CD4^+$ cells. Immune function was determined by their expression of immune checkpoint antibodies CTLA-4 and PD-1 and cytokine production. Inhibitors of CTLA-4 and PD-1 have been developed as cancer treatments [43], but have been suggested for use in sepsis as they can restore T cell function [34]. TNF, IFN-γ and IL-17A were selected as pro-inflammatory cytokines and IL-10 as an anti-inflammatory cytokine.

B cells are characterised as $CD19^+CD3^-$ cells. Their function was evaluated by IL-6 and IL-10 production.

Dendritic cells are characterised as $CD11c^+CD3^-CD14^-CD19^-CD20^-CD56^-CD16^-$ cells. Immune function was studied using HLA-DR expression for antigen-presenting function, PD-L1 expression immune checkpoint antibody, production of TNF and IL-6 for pro-inflammatory cytokine, and IL-10 for anti-inflammatory cytokine production.

Monocyte immune function was defined by the same functional antibodies as dendritic cells, but defined as $CD33^+CD14^+CD3^-CD19^-CD56^-$ cells.

a. CD4+ helper T cells

Acquisition   Single cells   Single cells

Mono- and
lymphocytes   Live cells   CD3+   CD4+

b. B cells

Acquisition   Single cells   Single cells

Mono- and
lymphocytes   Live cells   CD19+CD3-

c. Dendritic cells

Acquisition   Single cells   Single cells

Mono- and
lymphocytes   Live cells   Lin-   CD56-

d. Monocytes

Acquisition   Single cells   Single cells   Monocytes

Live cells   CD3-CD19-   CD56-   CD33+CD14+

Figure 2-3: Gating strategy used for (a) CD4+ helper T cells, (b) B cells, (c) dendritic cells and (d) monocytes.

Laser	Filter	Fluorochrome	CD4+ helper T cells	B cells	Dendritic cells	Monocytes
Violet	440/50	PB	PD-1		CD16	
	512/25	Aqua	Live/dead	Live/dead	Live/dead	Live/dead
	603/48					
	710/50	BV711				CD33
Blue	530/30	FITC	TNF		Lin-2	CD14
	590/40					
	695/40	PerCP-EF710	CTLA-4	IL-6	IL-6	IL-6
Yellow	585/16	PE	IL-10	IL-10	IL-10	IL-10
	620/15	PE-CF594	CD4	CD3	CD11c	HLA-DR
	695/40					
	780/60	PE-CY7			PD-L1	PD-L1
Red	670/14	APC	IL-17A		TNF	TNF
	720/30	AF700	IFN-g			CD56
	780/60	APC-Cy7	CD3	CD19	HLA-DR	CD3/CD19

Table 2-1: Flow cytometry panels for immune cell function of CD4+ T helper, B, dendritic cells and monocytes.

PBMCs were isolated from buffy coats from two healthy volunteers. Freshly isolated PBMCs were seeded in round bottom 96-well plates at a density of 1×10^6 cells/well. PBMCs were stimulated with LPS (100 ng/ml), *E. coli* and *S. aureus* (PBMC:bacterium ratio 5:1) and treated with palmitate (0.1 and 0.4 mM), butyrate (0.6 and 1.8 mM) and alpha-linolenic acid (0.1 and 0.3 mM) for 4 hours and overnight.

Four hours prior to the end of stimulation, 5 µg/ml brefeldin A (Biolegend, San Diego, California, USA) was added to block cytokine secretion. All staining procedures were performed at room temperature in the dark. Panels are shown in Table 2-1. At the end of incubation, cells were pelleted and stained for 20 minutes with live/dead fixable aqua (ThermoFisher Scientific) diluted in PBS. The stain was removed and Fc receptors were blocked with 5 µl beriglobin (BD Biosciences) to prevent non-specific binding. Cells were then stained with surface markers in cell staining media (CSM, PBS with sodium azide, BSA and EDTA) for 20 minutes. Cells were washed with CSM and fixed with 2.4% formaldehyde in PBS for 20 minutes. Cells were washed with CSM + 0.3% saponin (CSM-S) and stained for 20 minutes

with antibodies for intracellular cytokines. Cells were washed twice with CSM prior to flow cytometry (Attune NxT, ThermoFisher Scientific). A minimum of 300,000 events per measurement were read. Cell populations were identified using a sequential gating strategy (Figure 2-3). After the exclusion of doublets and debris, live cells were selected, then gating for specific cell populations was applied and geometric mean later assessed using FlowJo software version 10.0 (Tree Star Inc, Ashland, OR, USA).

No statistical analyses were performed due to low number of replicates (n=2).

2.6   Mitochondrial respirometry

The 'mitochondrial stress test' was used to assess different components of mitochondrial respiration in stimulated PBMCs treated with palmitate, butyrate and alpha-linolenic acid. The mitochondrial stress test uses various drugs to either inhibit or stimulate different components of the electron transport chain. A schematic overview of drugs used in this stress test is shown in Figure 2-4. Glutamine, pyruvate and glucose were added as respiratory substrates, so that substrates are not the limiting factor. Glutamine is an important substrate for immune cells, and pyruvate an important substrate in glycolysis. Glutamine gets converted to glutamate which can then enter the TCA cycle, whereas pyruvate enters the TCA cycle after conversion to acetyl-CoA.

After basal respiration measurements, oligomycin is injected. This is an inhibitor of ATP synthase (complex V) and decreases electron flow through the electron transport chain and hence mitochondrial respiration that is measured as the OCR (oxygen consumption rate). The decrease in OCR is linked to cellular ATP production.

The uncoupling agent FCCP is then injected. FCCP shuttles protons across the inner mitochondrial membrane, dissipating the protonmotive force and driving maximal activity of the ETC in an attempt to maintain it [343]. Allowing proton re-entry into the matrix uncouples the respiratory chain from ATP synthesis. FCCP thereby causes

rapid oxidation of substrates. Electron flow is not inhibited, and oxygen consumption at complex IV reaches its maximum.

The difference between maximum respiration and basal respiration is calculated as spare respiratory capacity. This indicates the cell's ability to respond to increased energy demand or when under stress. This gives a measure of how closely the cell is respiring to its theoretical maximum and indicates cell fitness or flexibility. The lack of spare respiratory capacity does not indicate dysfunction *per se* as it can also indicate a higher ATP demand such as biosynthesis for proliferation.

This is then followed by injection of rotenone and antimycin A, inhibitors of complexes I and complex III, respectively. This combination blocks mitochondrial respiration, so any remaining oxygen consumption is non-mitochondrial, mostly that by cellular enzymes. In monocytes and leukocytes, non-mitochondrial respiration can contribute significantly to overall OCR [344], likely due to increased activity of NAD(P)H oxidases [345].
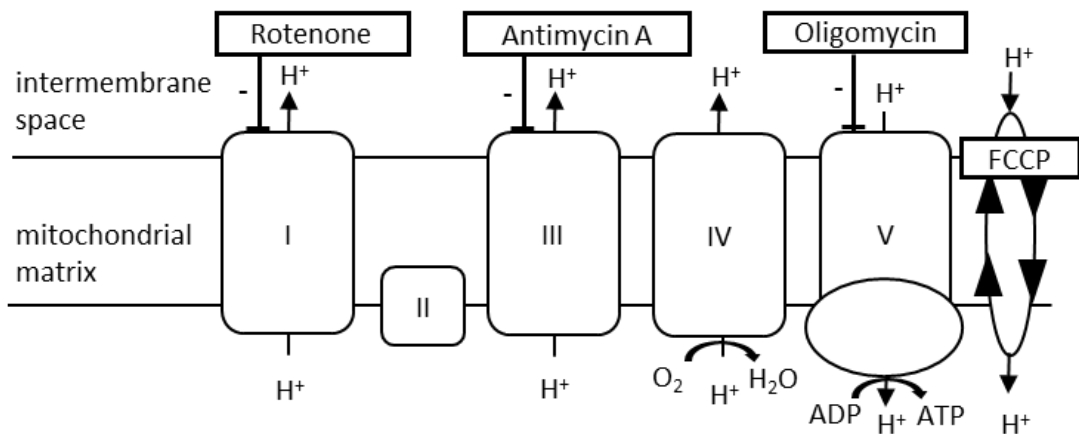
Figure 2-4: Schematic overview of drugs used to modulate the electron transport chain.

The Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used according to the manufacturer's protocol to measure the oxygen consumption rate (OCR) of human PBMCs.

PBMCs from six healthy volunteers were isolated and stimulated overnight as described earlier. They were then seeded in triplicate at a density of 300,000 cells per well. After overnight incubation, plates were centrifuged and supernatant was stored at -20°C for further cytokine analyses. Cells were resuspended in Seahorse XF DMEM medium (Agilent Technologies) supplemented with 5 mM glucose XF (Agilent Technologies), 1 mM pyruvate XF (Agilent Technologies) and 2 mM glutamine (Sigma-Aldrich) in specialised XF96 cell culture microplates (Agilent Technologies) pre-treated with Cell Tak (VWR international, Radnor, PA, USA). Plates were centrifuged at 200g for 1 minute without break and then incubated for 30 minutes at 37°C with no CO_2. Plates were then loaded onto the Seahorse analyser. Three baseline OCR measurements were taken for each well, after which oligomycin complex (2 µM, Sigma-Aldrich), carbonycyanide p-(trifluoromethoxy) phenylhydrazone (FCCP, 2 µM, Cambridge Bioscience, Cambridge, UK), antimycin A and rotenone (1 µM, Insight Biotechnology, Wembley, UK) were sequentially injected. Three OCR measurements were taken after each injection. Hoechst dye (ThermoFisher Scientific) was added to normalise OCR measurements according to cell number using the ImageXpress Imaging System (Molecular Devices, San Jose, CA, USA).
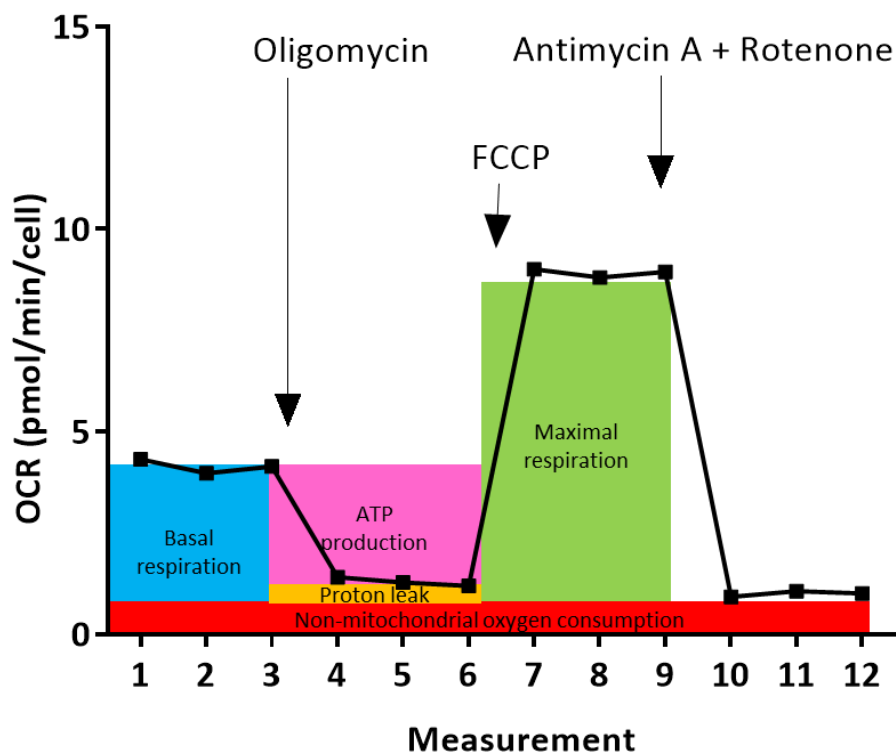
Figure 2-5: Visual representation of mitochondrial respiratory parameters.

To calculate OCRs for basal respiration, proton leak, ATP production, maximal respiration and non-mitochondrial respiration, the area under the curve (AUC) prior to the next injection was calculated. Parameter values were calculated as per Table 2-2 and Figure 2-5.

For statistical analyses, AUCs values per mitochondrial parameter of fatty acid treated groups were compared to the control group (being stimulus without fatty acid) for each individual stimulus. The non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's correction for multiple comparisons was used.

Table 2-2: Calculations used for mitochondrial respiratory parameters.

Parameter value	Equation
Non-mitochondrial respiration	AUC after antimycin A/rotenone injection
Basal respiration	(AUC before oligomycin injection) – (non-mitochondrial respiration)
Maximal respiration	(AUC after FCCP injection) – (non-mitochondrial respiration)
Proton leak	(AUC after oligomycin injection) – (non-mitochondrial respiration)
ATP production	(AUC before oligomycin injection) – (AUC after oligomycin injection)
Spare respiratory capacity	(Maximal respiration) – (basal respiration)

2.7 Characterisation of long-term rat model of faecal peritonitis

A long-term fluid-resuscitated rat model of faecal peritonitis was used for *in vivo* studies. All animal experiments were performed under a Home Office Project Licence (PPL 70/7029) and local UCL Ethics Committee approval. All experiments were performed in accordance with relevant guidelines and regulations.

2.7.1 Instrumentation long-term sepsis model

Male Wistar rats (Charles River, Margate, UK) weighing between 300-450g underwent instrumentation and echocardiography under isoflurane anaesthesia (Baxter Healthcare, Thetford, Norfolk, UK) administered by a vaporiser (Vet-Tech Solutions, Congleton, Cheshire, UK). Anaesthesia was induced by placing the animal in an induction chamber and spontaneously breathing 5% isoflurane. Maintenance anaesthesia was then achieved using 2% isoflurane with the rats spontaneously breathing the isoflurane through a nose cone.

A rectal probe was inserted for continuous temperature monitoring and connected to a heated mat (TES Electrical Electronic Corp, Taipei, Taiwan) for maintenance of body temperature at 36.5 – 37.5°C during anaesthesia.

Hair was removed from the neck and chest using depilatory cream to clear these areas prior to instrumentation. The depth of anaesthesia was assessed by toe and tail pinch. A subcutaneous injection of 0.025 mg/kg buprenorphine (Vetergesic, Reckitt Benckiser, Slough, UK) was given to provide long-acting analgesia. The skin was cleaned with disinfectant, followed by a small incision of 2 mm length at the nape of the neck. A 2 cm vertical incision in the anterior neck followed by tissue dissection was used to access the right internal jugular vein and he left common carotid artery. These vessels were then cannulated with 0.96 mm outer diameter PVC tubing (Scientific Commodities, Lake Havasu City, CA, USA). The tubing was secured within the vessel to a depth of 2-3 cm using 3-0 silk sutures. The other end was tunnelled subcutaneously to emerge at the small opening made at the nape of the neck. The tubing was then connected to a dual channel swivel tether system (InsTech Solomon, Plymouth Meeting, PA, USA) and flushed through. The skin incision sites were sutured with 2-0 sutures and cleaned with disinfectant. Another subcutaneous injection of 0.025 mg/kg buprenorphine was given to provide analgesia prior to recovery. The animals were then removed from anaesthesia and allowed to awaken in separate metabolic cages connected to a Comprehensive Lab Animal Monitoring System (CLAMS Oxymax, Columbus Instruments, Ohio, PA, USA). The vascular cannulae were connected to a swivel-tether system attached to a balancing arm. This allows free movement of the animal within its cage, with access to food and water *ad libitum* (Figure 2-6).
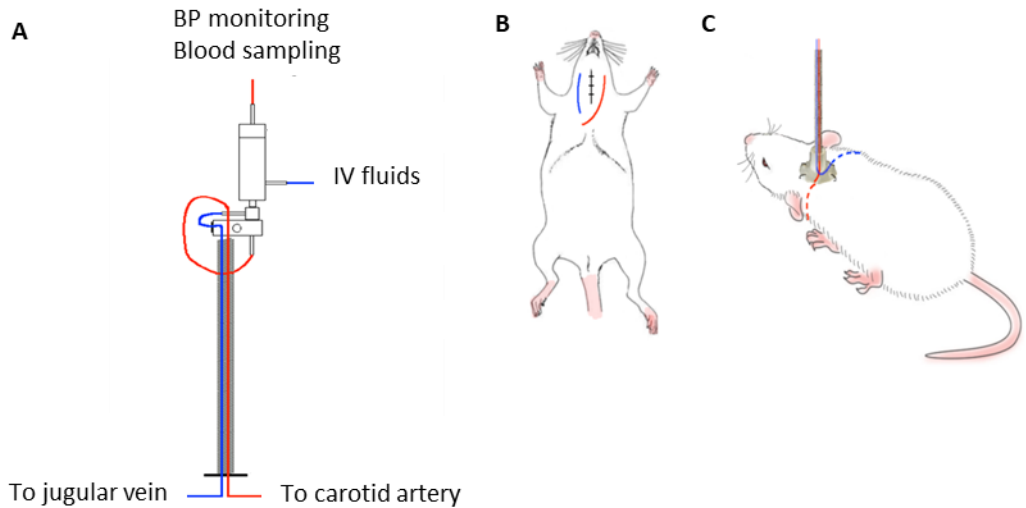
Figure 2-6: Instrumentation of rats with arterial and venous lines and usage of tether system.

(A) The arterial line (shown in red) is attached to the top of the swivel. The venous line (shown in blue) is attached to the side. The swivel can turn through 360 degrees, allowing the animal to freely move. (B) The arterial line (shown in red) is inserted into the carotid artery. The venous line (shown in blue) is inserted into the jugular vein. (C) Both lines emerge from an incision made at the nape of the neck and are connected to the swivel system.

The vascular cannulation enables continuous blood pressure monitoring and blood sampling through the arterial line and fluid resuscitation through the venous line. The carotid arterial line was connected to a pressure transducer (Memscap, Skoppum, Norway) for blood pressure monitoring into a Powerlab system (AD Instruments, Chalgrove, Oxon, UK) and flushed with 0.3 ml/h heparinised saline (2000 IU/L) (Baxter Healthcare) to prevent clotting. The jugular venous line was connected to an infusion pump with a 1:1 ratio of 5% glucose (to prevent hypoglycaemia) and sodium lactate solution (a crystalloid solution closely isotonic to blood, containing sodium, chloride, potassium and calcium ions, and lactate) given for continuous fluid resuscitation (10 ml/kg/h) starting at 2 hours post-induction of sepsis.

2.7.2 Sepsis insult

2.7.2.1 Stool preparation

Stool samples from three healthy human volunteers were obtained and pooled using a protocol developed by Prof. Michael Bauer and Dr. Ralf Claus (Jena University Hospital, Jena, Germany). In brief, nitrogen was flushed through the stool for 5 minutes and thioglycollate broth added in the same volume as the stool weight. Barium sulphate and glycerol (10% of total weight), and 200 µl catalase were then added. The stool mixture was suspended into a faecal slurry for about 10 minutes under nitrogen. Aliquots of 50 ml were then prepared under nitrogen to prevent segregation. This was frozen overnight to -20°C and stored at -80°C for long-term storage. Some of the slurry was further divided into 1 ml aliquots and stored at -80°C, enabling an identical septic insult to be given to each animal.

2.7.2.2 Induction of sepsis

Sepsis was induced in the rat by an intraperitoneal (i.p.) injection of faecal slurry using a 19-gauge needle injected into the right lower quadrant of the abdomen. For this purpose, a 1 ml vial of slurry was diluted into 7 ml n-saline and injected according to the weight of the animal. Successful injection into the peritoneum with generation of a peritonitis was confirmed post-mortem.

Sham operated control animals received no i.p. injection to avoid accidental bowel perforation. Naïve control animals got neither surgery nor an i.p. injection.

2.7.3 Clinical severity scoring

A clinical severity score was performed at least 4 times daily to assess the animal's level of (dis)comfort by assessing its general appearance, behaviour, and clinical signs (Table 2-3). It was developed in conjunction with the Biological Services Unit of UCL and has been approved by both the UCL vet and the Home Office Inspector. When animals score 2 points, monitoring is increased to every 2 hours. If no clinical improvement is seen in 8 hours, the animal is culled to not exceed the agreed severity limit. If the animal scores ≥3, it is culled immediately.

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 at touch, MABP >90 mmHg, slight panting
2	Cold at touch, MABP 75-90 mmHg, laboured breathing
3	Cold at touch, MABP <75 mmHg, abdominal breathing
Score	Other observations
4	Chronic diarrhoea (over 48 hours), coagulopathies (blood in urine, mouth, faeces)
4	Pale mucous membranes
4	Paralysis, ataxia, convulsions
4	Vocalisation
4	Large/ulcerated solid mass, untreatable skin wounds
Score 0	Normal
Score 1	Increase monitoring (minimum twice a day) provide additional support (mashed food etc.)
Score 2	Increase monitoring (minimum 4 times a day), provide additional support (mashed food, fluids, additional nesting), cull if no improvement in 48 hours
Score 3	Critical animal, increase monitoring (minimum every 4 hours), provide additional support (mashed food, fluids, nesting), continue monitoring overnight or cull if no improvement at the end of the day
Score 4	Euthanasia

Table 2-3: Sepsis score chart.

2.7.4   Survival study

A survival study was performed to identify the dose of faecal slurry insult that would generate a desired mortality of 30-40% at 72 hours, as this reflects an approximate mortality rate of sepsis from faecal peritonitis in humans [346]. However, unlike human patients, this animal model gets neither source control of the sepsis nor antibiotic administration. So, other than fluid resuscitation and analgesia, this follows a more natural course of events of a septic insult. Long experience with the model shows that those animals that die usually do so between 18-40 hours post-insult, whereas those animals surviving beyond this are showing signs of recovery by the end of the study at 72 hours, with increased alertness, activity, feeding and improved appearance.

The vessel cannulation, housing, clinical scoring and measurements were performed as described above. Increasing doses (4, 5, 6 and 7 µl/g body weight) of faecal slurry were used. Animals were culled at 72 hours or according to the clinical scoring protocol. Figure 2-7 gives a schematic overview of this survival study.

2.7.5   Blood sampling

Pre-mortem blood samples were taken by direct heart puncture. Up to 12 ml blood can be obtained by this approach. Blood was taken with a syringe pre-rinsed with heparin to prevent clotting. Tubes were centrifuged for 1 minute at 10,000 rpm. The separated plasma layer was pipetted into Eppendorf tubes which were then placed into liquid nitrogen prior to storage in a freezer at -80°C.

Peritonitis was confirmed visually at post-mortem, identifying evidence of a generalised inflammatory peritonitis response (purulent intraperitoneal fluid excess, abscess formation etc.).

2.7.6   Metabolic monitoring

Animals were housed in metabolic cages and connected to the CLAMS Oxymax system that continuously supplies fresh room air to the animal's cage and monitors oxygen and carbon dioxide concentrations at the inlet and outlet ports of the cage. Four cages could be monitored simultaneously. An oxygen sensor generates small

amounts of electricity when in contact with oxygen. This electrical signal is sensed through the circuity built into the CLAMS system. Carbon dioxide is sensed by single beam non-dispersive infrared light. The system was stabilised for at least 3 hours before the onset of each experiment while sensors were calibrated using a gas mix containing 20.5% O_2 and 0.5% CO_2.

O_2 consumption and CO_2 production were calculated by the difference between inlet and outlet flows to generate whole body values of oxygen consumption (VO_2) and carbon dioxide production (VCO_2), and thus computation of the RER.

For data analyses, average values per hour were taken. Multiple unpaired T-tests were performed using Holm-Sidak method for multiple comparisons to compare sham animals, septic survivors and septic non-survivors.
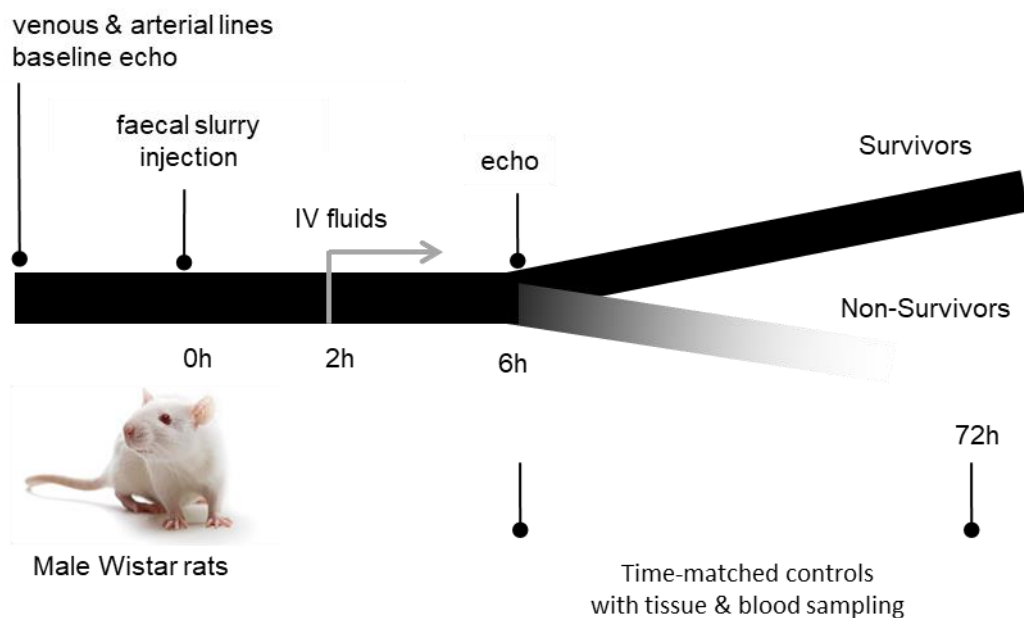
Figure 2-7: Survival study using the 3-day faecal peritonitis model.

After baseline echocardiography, rats were instrumented with tunnelled venous and arterial lines and faecal slurry then injected. Two hours later, IV fluids infusion was started. At six hours post-sepsis induction, a blood sample was taken and echocardiography performed. Further blood samples were taken at 24 hours and 72 hours, or at the time of death.

2.7.7 Echocardiography

Six hours after sepsis induction, transthoracic echocardiography (TTE) (Vivid-I, GE Healthcare, Bedford, UK) was performed to evaluate cardiac function. The animal was removed from its cage, and anaesthesia induced as described previously. The animal was placed on a heating mat with maintenance anaesthesia set to 1.5% isoflurane for 5-10 minutes. Temperature was monitored during the whole procedure.

Pulsed wave Doppler was used to measure aortic blood flow. The direction of blood flow was confirmed using colour Doppler. Heart rate (HR) was calculated by measuring the time between the start of each Doppler waveform over 4 consecutive waveforms.

The area under each waveform (i.e. cardiac contraction) is the velocity-time integral (VTI). The average VTI of six beats was taken to account for heart rate variability with respiration.

Stroke volume (SV) could then be calculated as:

$$SV = 0.25 \, \pi * d^2 * VTI$$

With 'd' being the aortic diameter which was set at 25 mm [347].

Cardiac output (CO) was calculated as:

$$CO = SV * HR$$

To measure respiratory rate, M-mode ultrasound of the diaphragm was used, using at least six consecutive respiratory cycles.

For statistical analyses, the non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to compare sham rats, septic survivors and septic non-survivors.

2.8 *In vivo* butyrate infusion study

To determine whether fatty acids affect immune and mitochondrial function and thus survival, butyrate infusion was used as a treatment in the previously described animal model of faecal peritonitis.

2.8.1 Drug safety studies

To test the tolerability and safety, a sham operated rat was kept anaesthetised and under continuous arterial blood pressure monitoring. Stepwise increased doses of sodium butyrate dissolved in saline (0, 0.1, 0.3, 1, 3 and 10 times intended drug dose of 0.6 g/kg) were given intravenously (IV) at an infusion rate of 5 ml/kg over 5 minutes. The animal was monitored for a duration of 25 minutes and an arterial blood gas, an echo Doppler cardiac output measurement and a plasma sample were taken before continuing to the next dose.

As an additional way of testing drug safety, another sham operated animal was given the intended drug dose of 0.6 g/kg intravenously at an infusion rate of 5 ml/kg over 5 minutes. The animal was monitored for 90 minutes. Blood gas, echo Doppler measurement and plasma samples were taken every 15 minutes, while arterial blood pressure was continuously measured. After 90 minutes, another dose of 0.6 g/kg was given and again monitored for 90 minutes.

Tolerability and safety were further assessed using awake animals instrumented with an arterial and venous line (Figure 2-8). Animals were housed in metabolic cages, with continuous arterial blood pressure and metabolic monitoring. Two hours after surgery, animals were infused with 1:1 glucose/sodium lactate solution at an infusion rate of 10 ml/kg/h. Six hours after surgery, animals started receiving butyrate infusion at a rate of 0.6 g/kg/h for 90 minutes, whereas control animals continued receiving glucose/sodium lactate as control solution. The infusion rate maintained at 10 ml/kg/h. Butyrate infusion was increased to 1.2 and 2.4 g/kg/h after another 90 and 180 minutes, but maintaining the same volume rate of fluid infused . A clinical score, blood gas and plasma sample were taken before changing butyrate dosage. After completing the 2.4 g/kg/h dosage, an echo Doppler

measurement was taken under brief anaesthetic. Butyrate infusion was reduced to 0.6 g/kg/h and continued overnight. At 24 hours after induction of sepsis, clinical score, blood gas, plasma sample and an echo Doppler measurement were taken before culling the animals by cardiac puncture.
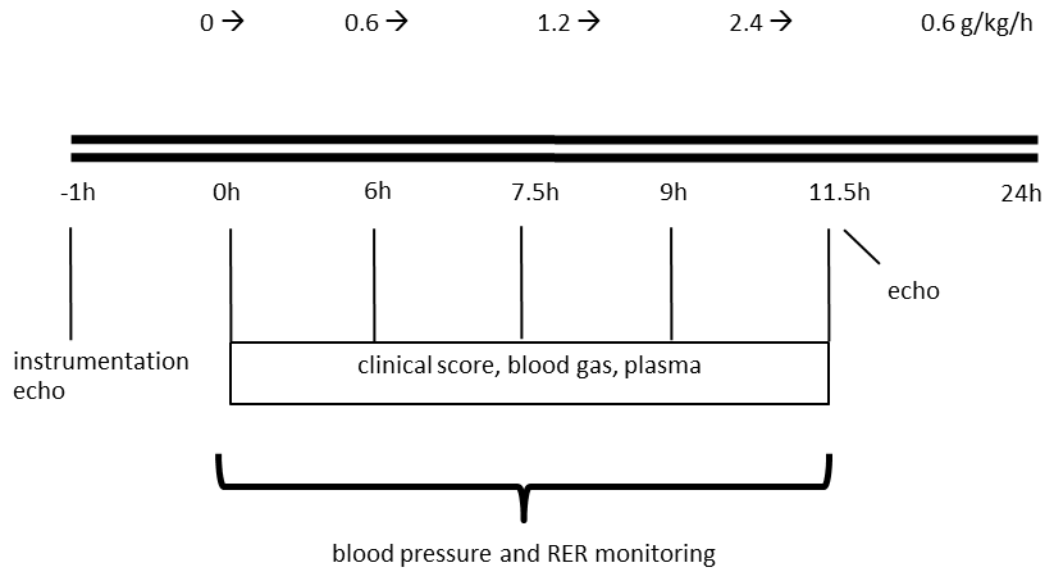
Figure 2-8: Experiment overview of tolerability and safety of butyrate in awake animals.

2.8.2 Butyrate infusion study

Animals were acclimatised overnight in metabolic cages. Animals were induced, and given an i.p. faecal slurry injection of 6 µg/kg to induce sepsis. Rats were then instrumented with a venous line and no arterial line. Animals were put back in the metabolic cages and given IV fluids (1:1 ratio of glucose/Hartmann's solution) from 2 hours after sepsis induction onwards. Echocardiography was taken six hours after sepsis induction, followed by a fluid bolus of 10 ml/kg over five minutes. Infusion of butyrate in glucose/sodium lactate was then started. Control animals received isovolumic and isocaloric glucose/Hartmann's solution. Twenty-four hours after sepsis induction, echocardiography was performed, and tissue and blood plasma were sampled. Naïve rats did not receive any treatment. The spleen was harvested for splenocyte isolation for further analyses. An experiment overview is shown in Figure 2-9.
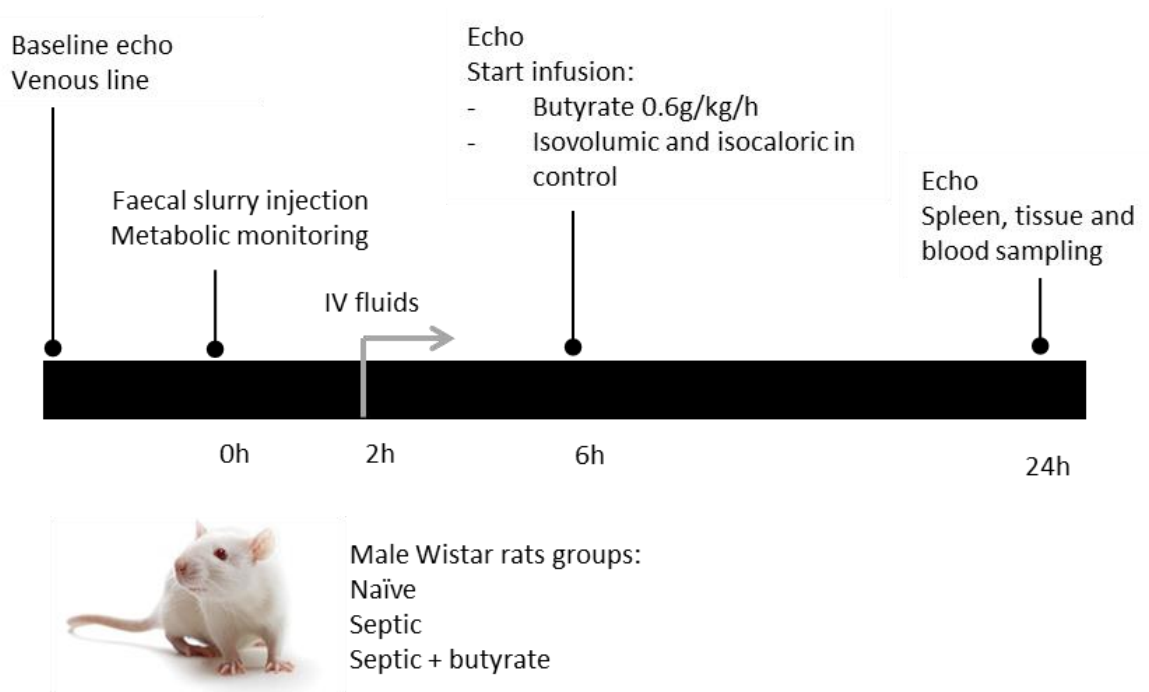
Figure 2-9: Experiment overview overnight *in vivo* butyrate infusion.

2.8.3 Isolation of rat splenocytes

As a limited number of circulating immune cells can be obtained per animal, especially when septic as lymphocyte and monocyte counts generally fall, this may prove limiting on *ex vivo* experiments. Either more animals need to be utilised or, preferably, an alternative immune cell can be investigated to reduce animal use. We thus sought to isolate fresh splenocytes from the rat.

After sacrificing the animal by cardiac puncture, the complete spleen was isolated and put in a Petri dish filled with fridge-cold culture medium. The spleen was broken in pieces in the Petri dish and then passed through a 100-μm cell strainer to obtain a single cell suspension by crushing with a 5 ml syringe and collecting the cell suspension in 5 ml culture medium. Culture medium was added on top of the strainer to keep the tissue under medium throughout. The cell suspension was centrifuged at 4°C, 130 RCF for 5 minutes. Cells were re-suspended in 15 ml 1x NH_4Cl lysing solution, incubated at room temperature for 10 minutes and centrifuged again to lyse and wash out erythrocytes. Splenocytes were washed twice in 15 ml PBS and re-suspended in culture medium.

For freezing purposes, cells were resuspended in freezing medium (filtered FBS with 10% DMSO). Splenocytes were aliquoted into cryogenic storage vials and frozen slowly at 1°C/minute to -80°C by placing the vials in an insulated box, followed by long-term storage at -80°C.

2.8.4 Mitochondrial respiration

Freshly isolated splenocytes were resuspended in Seahorse medium supplemented as described before and seeded in triplicate at a density of 300,000 cells/well. Plates were processed and data analysed as described in section 2.6.

For statistical analyses, the non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to compare AUC values of mitochondrial parameters of splenocytes isolated from naïve, septic butyrate treated and septic control animals.

2.8.5 Flow cytometry

Directly after isolation, staining protocol for cytokine production, mitochondrial ROS and mitochondrial membrane potential was started (panels in Table 2-4). Cells were resuspended in tubes with HBSS and stained with fixable violet live/dead (Thermo Fisher Scientific), CD3 (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD4 (BD Biosciences) MitoSOX red (2.5µM, ThermoFisher Scientific) or TMRM (25 nm, ThermoFisher Scientific) were added for measuring mitochondrial ROS and mitochondrial membrane potential, respectively. Cells were incubated for 20 minutes at 37°C in 5% CO_2 / 95% O_2 prior to loading on a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences). For cytokine analyses, cells were fixed and permeabilised after surface staining using fix/permeabilize solution (BD Biosciences) for 20 minutes at room temperature in the dark. Cells were washed with permeabilization/wash buffer (BD Biosciences) and stained with TNF (ThermoFisher Scientific) and IL-10 (BD Biosciences) for another 20 minutes at room temperature in the dark prior to loading on flow cytometer.

A minimum of 5000 events/measurement were read. Cell populations were identified using sequential gating strategy (Figure 2-10). After the exclusion of

doublets and debris, live cells were selected and Th2 cells were identified using CD3 and CD4 antibody and geometric mean assessed using FlowJo version 10.0 (Tree Star Inc, Ashland, OR, USA).

For statistical analyses, the non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to compare geometric means of splenocytes isolated from naïve rats, septic survivors and septic non-survivors.

Table 2-4: Flow cytometry panels for splenocytes.

Laser	Filter	Fluorochrome	Cytokine panel		Mitochondrial panel	
			Target	Clone	Target	Clone
Violet (405nm)	450/50	violet	live/dead		live/dead	
	525/50					
	605/12					
	710/50	BV711	CD4	OX-35	CD4	OX-35
	780/60					
Blue (488nm)	488/10	FITC	TNF	TN3-19.12		
	530/30					
	695/40					
Yellow (561nm)	585/15	PE	IL-10	A5-4	TMRM/mitoSOX	
	610/20					
	670/30					
	710/50					
	780/60					
Red (640nm)	670/14					
	730/45					
	780/60	APC-Vio770	CD3	REA223	CD3	REA223
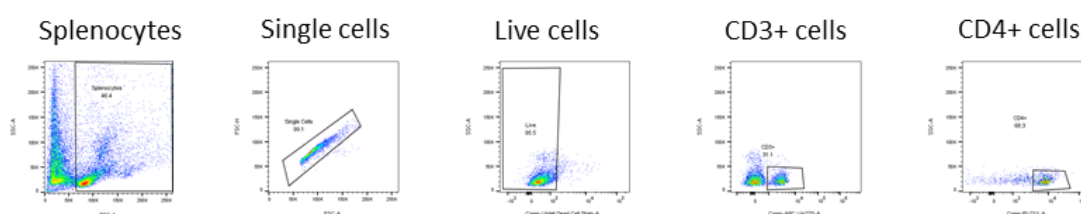
Figure 2-10: Gating strategy butyrate infusion study.

2.8.6   Splenocyte stimulation

Freshly isolated rat splenocytes were seeded at a density of 300.000 cells per well and stimulated overnight with LPS (100 and 1000 ng/ml) and phorbol 12-myristate 13-acetate (PMA, 200 nM). The next day, plates were centrifuged and supernatant carefully collected and stored at -20°C for cytokine analyses.

2.9   Enzyme-linked immunosorbent assays

IL-6, IL-10 and TNF secretion was measured in PBMC and whole blood stimulation supernatant, rat plasma samples and in supernatant of stimulated rat splenocytes by enzyme-linked immunosorbent assays (ELISAs, BD Biosciences and R&D Systems, Minneapolis, MN, USA) according to the manufacturers' protocol. Briefly, Maxi-sorb 96 wells plates (ThermoFisher Scientific) were coated with coating antibody and incubated overnight at 4°C. Plates were washed three times with wash buffer and blocked for an hour with assay diluent. Plates were washed, samples and standards were added and incubated for two hours at room temperature. Plates were washed and incubated for an hour at room temperature with detection antibody plus streptavidin-HRP. Plates were washed and incubated with substrate reagent (BD Biosciences) for 20 minutes at room temperature in the dark. Stop solution was added and absorbance was measured at 450 nm.

For data analyses, averages of three technical ELISA replicates (with exception of outliers) were taken. Fatty acid treated groups were compared to the control group for each stimulus for PBMC and whole blood stimulation supernatants. For butyrate infusion studies, naïve, butyrate treated and butyrate control animals were compared. The non-parametric one-way ANOVA Kruskal-Wallis test was used with Dunn's correction for multiple comparisons.

2.10   Statistical analyses

Graphpad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical analyses and graphs. Only non-parametric tests were performed due to a low n numbers. Data are presented as median (interquartile range). A p-value <0.05 was taken as statistically significant.

Chapter 3   The impact of fatty acids on immune function in an *in vitro* model of sepsis

3.1   Introduction

Depending on their hydrocarbon chain lengths, number, position and orientation of their double bonds, fatty acids have differential effects on immune function. An *in vitro* model of sepsis using human PBMCs and whole blood was developed to gain insight as to the different immunomodulatory effects of fatty acids with different chemical structures. Butyrate was chosen as a short-chain fatty acid, whereas palmitate and alpha-linolenic acid are both long-chain fatty acids, but saturated and unsaturated respectively.

As sepsis pathology involves different inflammatory phases as described in section 1.3, the clinical impact of fatty acids may differ depending on the patient's immune status. Therefore, I decided to use two different stimulation durations, namely 4 hours to represent early sepsis, and overnight to simulate late sepsis.

3.2   Results

3.2.1   Fatty acid dose finding study

A dose finding study was performed to determine what concentration of stimulus and fatty acid could be used to induce an inflammatory response, but remaining cell viability. Tested fatty acid concentrations are based on the physiological range [338, 339] and 3-fold elevated as in our septic rat model (unpublished data)). Concentrations of stimuli are based on a combination of literature research and experience within the lab.

Figure 3-1 shows the cytotoxicity results of PBMCs stimulated for 4 hours or overnight with LPS, EC and SA and treated with various concentrations of palmitate, butyrate and alpha-linolenic acid. Viability was not impaired in any of the conditions, with the exception of 24 hour stimulation with 2.2 mM palmitate (Figure 3-1). This concentration of fatty acid was also difficult to administer due to its poor

solubility in aqueous solutions. It was therefore decided to use lower concentrations of palmitate (0.1 and 0.4 mM) for future experiments.

For butyrate and alpha-linolenic acid, average and 3-fold plasma concentrations were chosen: 0.6 and 1.8 mM for butyrate and 0.1 and 0.3 mM for alpha-linolenic acid.
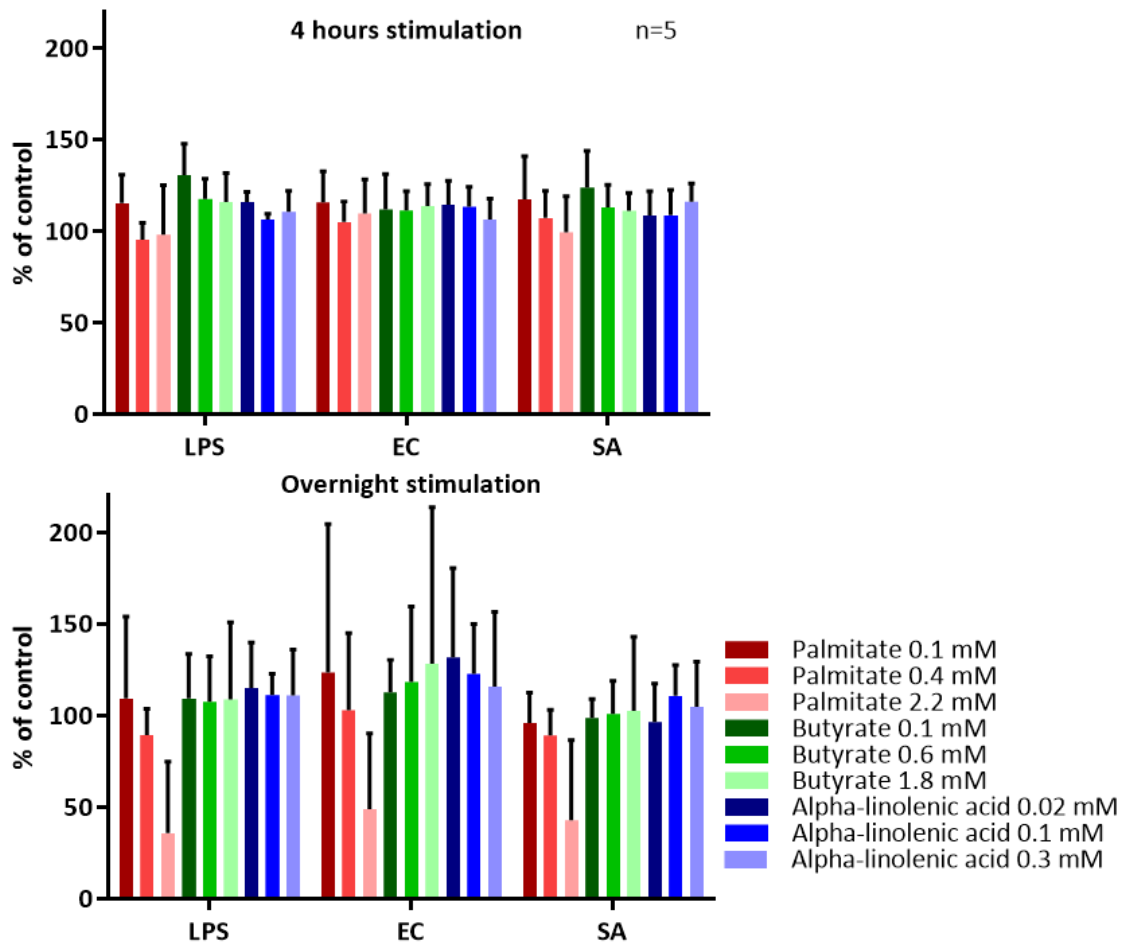
Figure 3-1: Cytotoxicity after 4 hours or overnight stimulation of PBMCs with stimuli and treatment of fatty acids (n=5). Data shown as percentage of control (stimulus without fatty acid) (mean+SD).

3.3 Cytokine secretion

3.3.1 Cytokine secretion by PBMCs

Cytokine secretion by stimulated PBMCs was measured in the cell supernatant. As expected, little or no cytokine secretion could be measured in unstimulated cells (data not shown). IL-10 secretion was not measured at 4 hours stimulation, as IL-10 production and secretion take longer to rise.

Results are displayed in Figure 3-2 below. Palmitate increased (albeit non-significantly) pro-inflammatory TNF secretion by PBMCs after 4 hours and more so after overnight stimulation. Little or no effect of palmitate was seen on pro-inflammatory IL-6 secretion, whereas a significant inhibitory effect on overnight IL-10 secretion upon EC stimulation was seen with 0.4 mM but not 0.1 mM.

Butyrate significantly decreased IL-10 secretion in all conditions and significantly decreased SA induced TNF secretion at 1.8 mM. Butyrate further non-significantly decreased TNF and IL-6 secretion in all other conditions.

Alpha-linolenic acid non-significantly decreased TNF secretion after both 4 hours and overnight stimulation. Alpha-linolenic acid however did not affect IL-6 secretion after 4 hours stimulation, though a trend towards increasing IL-6 secretion was seen after overnight stimulation.

Remarkably, all fatty acids suppressed secretion of anti-inflammatory IL-10, with the strongest effect seen for butyrate. These results display that palmitate, butyrate and alpha-linolenic acid have proinflammatory, anti-inflammatory and mixed effects on cytokine secretion, respectively.
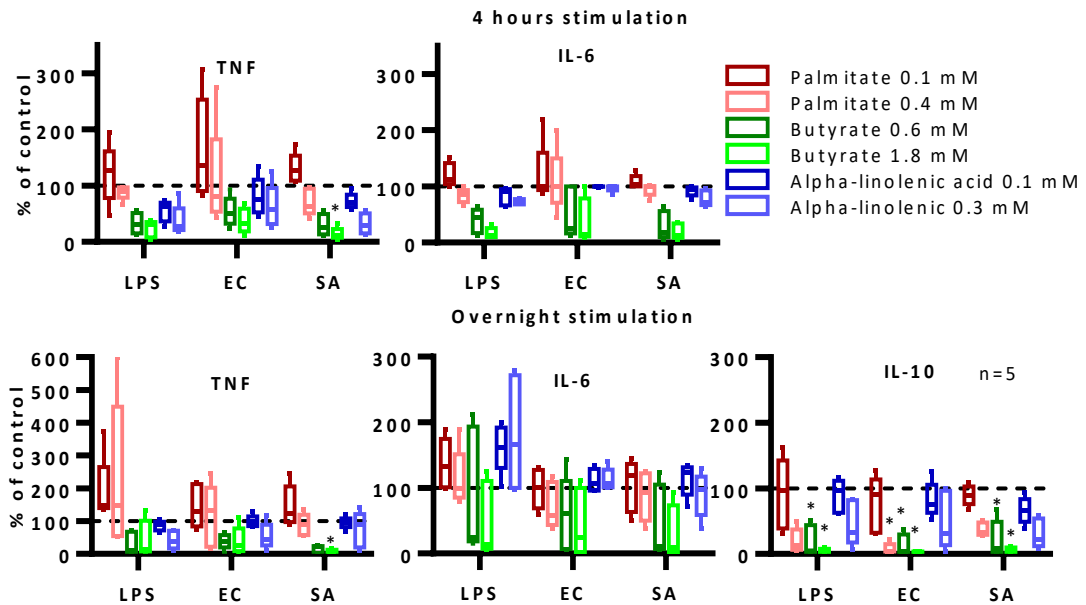
Figure 3-2: Cytokine secretion after 4 hours and overnight PBMC stimulation and treatment with fatty acids (n=5). Shown as percentage of control (mean±SD), being stimulus without fatty acid. *p<0.05.

3.4 Whole blood stimulation

Apart from monocytes, T, B and NK cells human blood contains erythrocytes, platelets and granulocytes and other components impacting the immune response. Therefore, immunological impact of fatty acids in whole blood was investigated.

None of the fatty acids affected LPS- nor EC-induced TNF secretion, though a trend towards reducing TNF secretion was shown for all fatty acids upon stimulation with SA (Figure 3-3).

Similar to PBMC stimulation (Figure 3-2), a trend towards increased LPS- and EC-induced IL-6 secretion was seen for alpha-linolenic acid in whole blood (Figure 3-3). However, in contrast to PBMC stimulation, palmitate reduced LPS- and EC- but not SA- induced IL-6 secretion in whole blood. Butyrate strongly inhibited LPS- and EC-induced IL-10 production, similar to what was seen with PBMC stimulation.

IL-1 receptor antagonist (IL-1RA) is a cytokine that can also bind to the IL-1 receptor but prevents IL-1 signal transduction [348]. Reduced secretion of IL-1RA compared to control was found in most conditions. This is consistent with an increase in secretion of the pro-inflammatory cytokine, IL-1β.
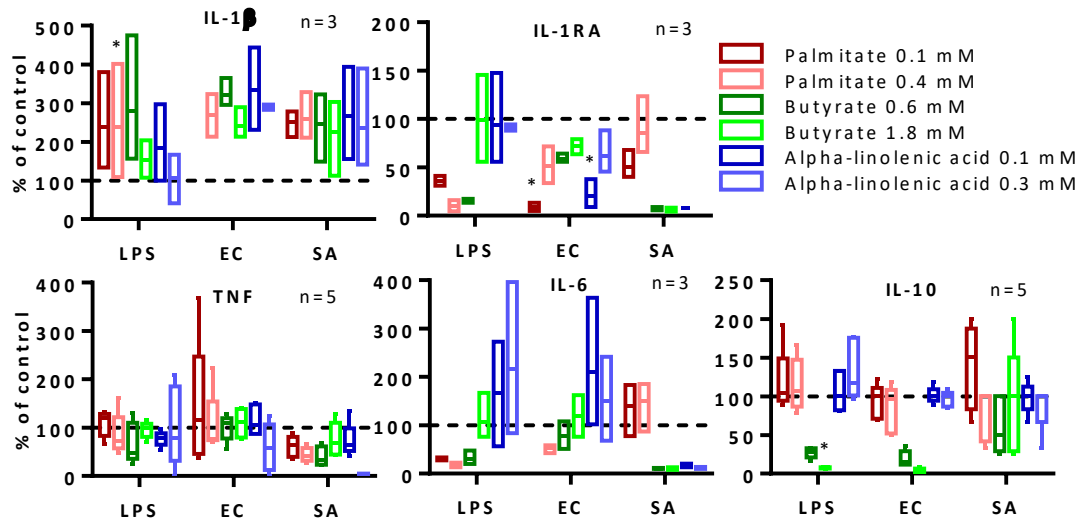
Figure 3-3: Cytokine secretion after overnight whole blood stimulation and treatment with fatty acids (n=3-5). Shown as percentage of control (mean±SD), being stimulus without fatty acid. *p<0.05.

3.5 Flow cytometry

Fatty acids impact on PBMC immune function by modifying the secretion of IL-6, IL-10 and TNF (Figure 3-2). This was measured by ELISA which measures total cytokine secretion of all cells and is not cell-specific. Flow cytometry is a widely used technique to study the characteristics of single cell populations. Here, flow cytometry was used to research the effects of fatty acids on CD4+ helper T cells, B cells, dendritic cells and monocytes, using the panels as described in Table 2-1. I chose to study all PBMCs except NK cells as these are limited in cell numbers and likely play a less significant role in sepsis. This experiment was only performed twice and served as a pilot study. Hence no statistical tests were performed and only general observations are described below.

A live/dead stain was used to assess cell viability after the incubation period. All PBMC incubations used for this study had a viability of ≥96% per well, with a few exceptions of ≥81% viability (data not shown). Of note, only the last 4 hours of cytokine production could be captured due to cytotoxicity effects of brefeldin A (used to block export of cytokines).

3.5.1 CD4+ helper T cells

Results on CD4+ helper T cells are shown in Figure 3-4. No effects of fatty acids on intracellular production of TNF and IFN-γ by CD4+ helper T cells were seen. Fatty acids did not impact on PD-1 expression (data not shown).

Palmitate 0.4 mM increased IL-17A production after 4 hours' stimulation, but reduced production after overnight stimulation. Butyrate 1.8 mM decreased EC- and SA-induced IL-17A production when stimulated overnight. However, no differences were seen between the unstimulated (control) group and LPS-, EC- and SA-stimulated groups in IL-17A production.

Overnight stimulation of PBMCs with EC and SA increased IL-10 production compared to unstimulated cells. Both palmitate 0.4 mM and butyrate 1.8 mM however reduced IL-10 production after overnight stimulation. This corresponds with the inhibitory effects of palmitate and butyrate (and alpha-linolenic acid) on IL-10 secretion seen in the PBMC supernatant study (Figure 3-2).

No differences in expression of the immune checkpoint, CTLA-4 was seen after 4 hours of stimulation, however expression was decreased by palmitate 0.4 mM and butyrate 1.8 mM in overnight EC- and SA-stimulated PBMCs (Figure 3-4).
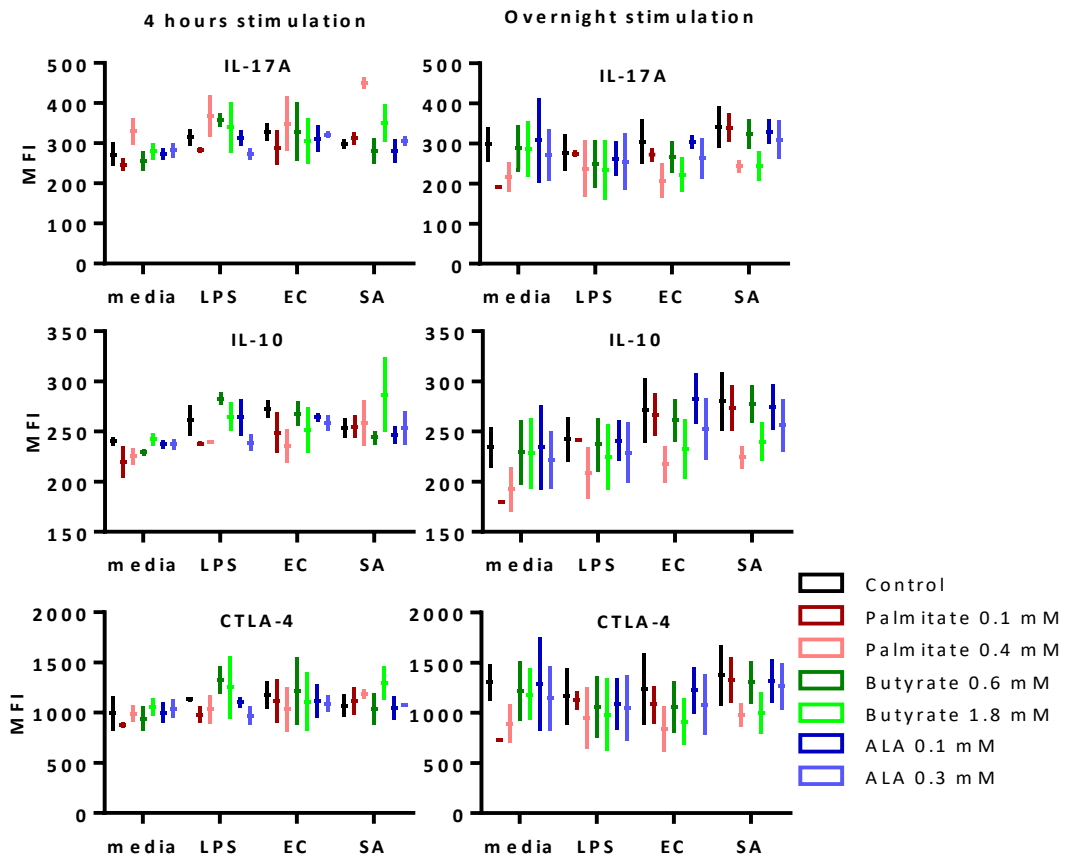
Figure 3-4: IFN-γ, IL-10 production and CTLA-4 expression by CD4+ T helper cells after 4 hours and overnight stimulation and treatment with fatty acids.

3.5.2   B cells

IL-6 and IL-10 production was higher in stimulated compared to unstimulated cells. Fatty acids did not impact upon IL-6 nor IL-10 production by B cells after 4 hours of stimulation. However, a decrease compared to control was seen after overnight stimulation with the addition of the lipids, with a suggestion of dose dependency (Figure 3-5).
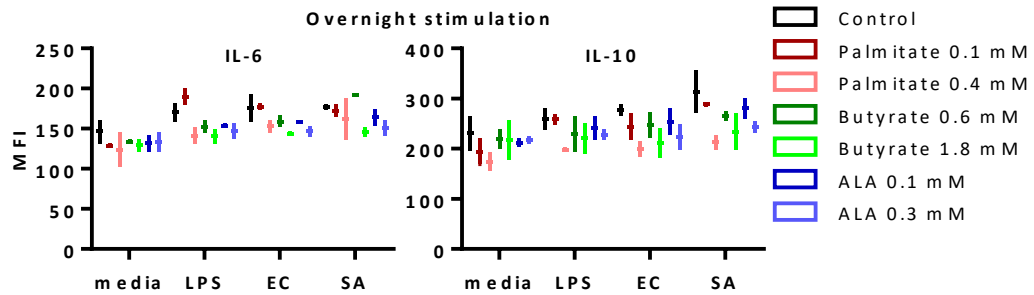
Figure 3-5: IL-6 and IL-10 production by B cells after overnight stimulation and treatment with fatty acids.

3.5.3   Dendritic cells

IL-6 production did not differ between unstimulated, LPS-, EC- and SA- stimulated groups. No impact of fatty acids was seen on IL-6 levels at 4 hours (data not shown). However, after overnight stimulation, palmitate 0.4 mM suppressed IL-6 production whereas butyrate and alpha-linolenic acid increased LPS- and EC-induced IL-6 production at all concentrations. Butyrate also increased SA-induced IL-6 production.

IL-10 production by dendritic cells was increased following LPS, EC and SA stimulation. Palmitate 0.4 mM decreased IL-10 production following overnight stimulation. There was no effect of either butyrate and alpha-linolenic acid on IL-10 production (Figure 3-6). The same trend was seen at 4 hours (data not shown).

HLA-DR expression by DCs increased upon stimulation from 4 hours onwards (data not shown) with the highest increase seen when PBMCs were treated with 0.4 mM palmitate (in both LPS and EC groups, but not with SA), and also when treated with 1.8 mM butyrate (LPS and EC groups).

No effect on immune checkpoint PD-L1 expression was seen after 4 hours (data not shown). At overnight incubation though, 1.8 mM butyrate increased PD-L1

expression in LPS-, EC- but not SA-stimulated dendritic cells. Stimulation with SA resulted in the highest PD-L1 expression, with reduced expression caused by palmitate at 0.4 mM (Figure 3-6).
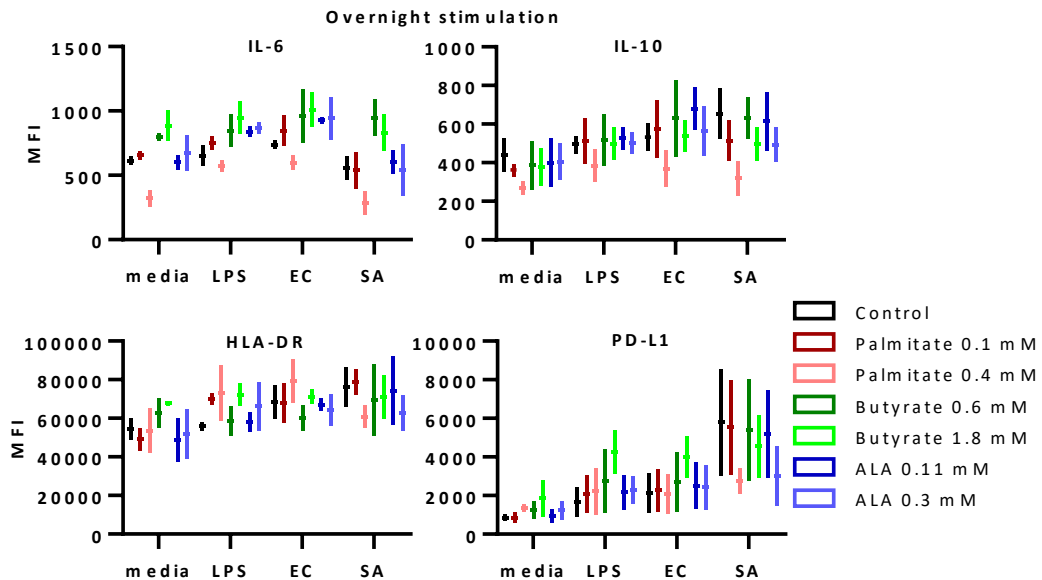
Figure 3-6: IL-6 and IL-10 secretion, and HLA-DR and PD-L1 expression by dendritic cells after overnight stimulation and treatment with fatty acids.

3.5.4 Monocytes

Butyrate (and alpha-linolenic acid to a lesser extent) inhibited LPS- and EC-induced IL-6 production in monocytes after 4 hours' stimulation (Figure 3-7). The opposite result was however found after overnight stimulation with 1.8 mM butyrate, with increased IL-6 production. 0.4 mM palmitate also appeared to decrease IL-6 production in after overnight stimulation.

LPS- and EC-stimulated TNF production increased at 4 hours but this was depressed in a concentration-dependent manner on addition of the lipids. 0.4 mM palmitate however decreased IL-10 production in monocytes after overnight stimulation.

PD-L1 was decreased after 4 hours of LPS and EC stimulation. An overall higher expression of PD-L1 was found after overnight stimulation, but this was suppressed in cells co-treated with 0.4 mM palmitate. No effect of fatty acids was however found on monocyte HLA-DR expression (data not shown).
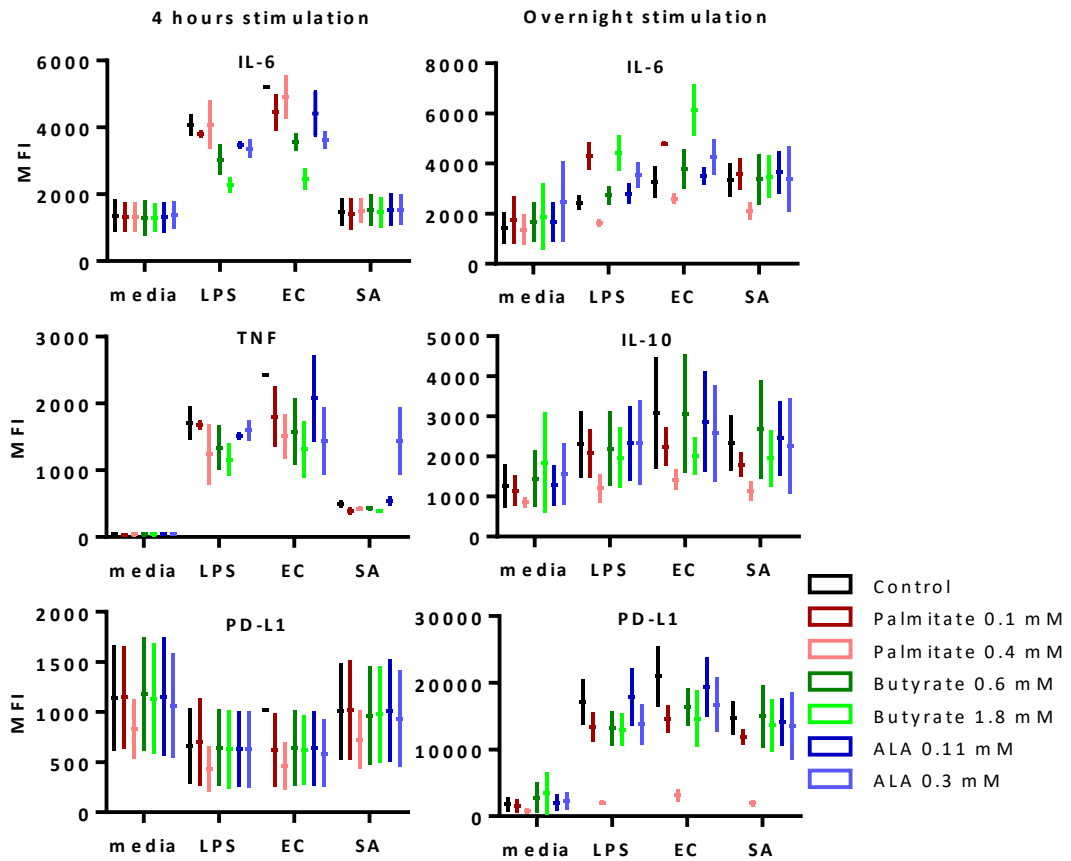
Figure 3-7: Production of IL-6, TNF and IL-10, and expression of PD-L1 by monocytes after 4 hours and overnight incubation and treatment with fatty acids.

3.6   Discussion

3.6.1   Cell viability

The MTT assay is used as a standard measure of viability. However, as it is based on the activity of mitochondrial enzymes to reduce the MTT to formazan, it is arguably not a true measure of cytotoxicity. Other limitations of the MTT assay are discussed

elsewhere [349-352]. However, this rapid method provides a relative although not absolute idea of cytotoxicity, with viability being later confirmed with flow cytometry.

No effect on toxicity was seen after 4 hours of incubation with any of the concentrations of fatty acids tested. However, after overnight incubation, palmitate 2.2 mM did show cytotoxicity (Figure 3-1) and this dose was subsequently abandoned. Palmitate is a surfactant, acting as a detergent, explaining its toxic effect at highest concentration. Most cell studies using palmitate use no more than 0.5 mM, and usually between 0.1-0.2 mM [353-356].

In the blood, solubilisation and efficient transport of long-chain fatty acids is enhanced by binding to serum albumin. This could explain why palmitate, one of the most prevalent fatty acids in humans [357], is soluble and non-toxic when concentrations are in normal range. *In vitro* studies often conjugate palmitate to albumin to overcome its poor solubility in aqueous solutions. However, as only unbound FFAs are accessible for cellular uptake, I chose not to conjugate palmitate to additional albumin although the cell culture medium did contain some. 10% fetal bovine serum, in medium results in an 0.25% increase in albumin concentration, and this may significantly alter unbound FFA concentrations and cellular effects [357].

Palmitate is suggested to induce cell death via incorporation into *de novo* synthesis of the lipid-signalling molecule ceramide, which may induce apoptosis [358]. Other mechanisms have also been proposed [356]. Using the MTT assay, Kong *et al.* showed a dose-dependent palmitate loss of cell viability in cardiomyocytes [359], inducing both necrosis and early apoptosis, loss of DNA content, and reducing mitochondrial oxidative capacity. Saturated (e.g. palmitate) but not unsaturated fatty acids induce cell death in various cell types (e.g. T cells, human aortic endothelial cells, hamster ovary cells) [356, 360]. Saturated fatty acids can also induce apoptotic death in skeletal muscle [361], hepatocytes [362] and pancreatic β cells [363].

In a hamster ovary cell model, palmitate (saturated) but not oleate (monounsaturated) induced ceramide-independent programmed cell death with annexin V positivity, increased caspase 3 activity and DNA fragmentation [356]. Palmitate-induced apoptosis occurred via ROS generation, which was seen after 5 hours' incubation with palmitate concentrations varying from 0.1-0.5 mM. In addition, as opposed to various unsaturated fatty acids, palmitate activates T cells and is involved in generation of ROS, lipid peroxidation, and increased secretion of pro-inflammatory cytokines with deleterious effects [360].

3.6.2 Cytokine secretion by PBMCs

The results shown in Figure 3-2 demonstrate that palmitate, butyrate and alpha-linolenic acid have proinflammatory, anti-inflammatory and mixed effects on cytokine secretion.

3.6.2.1 *Palmitate*

In this study, palmitate increased pro-inflammatory TNF and inhibited anti-inflammatory IL-10 secretion. Similar were observed in macrophage studies [354, 364], with B cells [365] and myoblasts [366]. LPS-stimulated PBMCs in the presence of palmitate resulted in increased IL-6 gene expression [367]. This is in accordance with the increase in IL-6 secretion I found upon overnight PBMC stimulation (Figure 3-2). Of note, IL-6 mRNA expression can be detected in whole blood 2-4 hours after stimulation, and IL-6 protein after 4-6 hours [368]. However, the literature is not totally consistent; one study found that palmitate increased T cell secretion of TNF, IL-6, IL-1β and IL-8, yet also IL-10 [360].

I found that TNF also increased upon palmitate exposure. Besides recruiting and activating immune cells, TNF is also involved in the production of various cytokines including IL-6.

In vivo studies also show pro-inflammatory effects of palmitate. PBMCs of healthy volunteers taking a 3-week high palmitate diet showed a higher secretion of the pro-inflammatory cytokine, IL-18 and a trend towards higher IL-1β secretion upon LPS stimulation compared to those taking a high oleate diet. The high palmitate diet

further resulted in elevated plasma IL-6 and IL-1β levels [369]. The same group also demonstrated that a higher palmitate to oleate ratio was associated with higher circulating concentrations of IL-6 and TNF and increased secretion of IL-1β, IL-18 and TNF by LPS-stimulated PBMCs [370, 371]. The authors suggested this was indicative of activation of both TLR4 and the NLRP3 inflammasome [369]. Increased production of pro-inflammatory cytokines after palmitate exposure is TLR4 dependent, as shown previously in monocytes [372] and PBMCs [373]. I did not study this mechanism but note that the TLR4 pathway may be most relevant for LPS and *E. coli* stimulation, as TLR4 is activated by Gram-negative bacteria.

Cell membrane composition has not been studied. Fatty acid composition of cell membranes determines immune cell function by affecting the neutrophil oxidative burst, phagocytic capacity of neutrophils and monocytes, lymphocyte proliferation and, to a lesser extent, cytokine production [374]. This has been excellently reviewed elsewhere [170]. The ratios of saturated fatty acids to PUFAs negatively correlate with these immune functions. For example, increased palmitate incorporation into T cell membranes was negatively associated with phagocytic capacity [374].

3.6.2.2   Butyrate

Figure 3-2 demonstrates that butyrate reduces TNF, IL-6 and IL-10 secretion at both 4 hours and overnight stimulation, for all infectious stimuli. Even though IL-10 is an anti-inflammatory cytokine, these data suggest an overall anti-inflammatory effect of butyrate, with general suppression of cytokine secretion. As butyrate is a strong inhibitor of NF-κB, this might be the underlying mechanism. NF-κB controls gene expression encoding pro-inflammatory cytokines, enzymes inducing inflammation, growth factors, immune receptors and heat shock proteins [375]. Activation of NF-κB is also involved in increased IL-10 production [376], suggesting inhibition may result in the opposite. Here, however, NF-κB activity has not been measured.

Other underlying mechanisms of suppression could involve inhibition of HDAC and binding to G protein-coupled receptors (GPRs) 41 and 43, later renamed as free fatty acid receptors FFAR3 and FFAR2, respectively. Butyrate is a broad HDAC

inhibitor, leading to hyperacetylation of both histone and non-histone proteins in transcription factors, thereby altering gene expression. FFAR2 is largely found on PBMCs and neutrophils [377, 378], but its exact role is still a topic of debate. However, concentrations needed for HDAC inhibition and FFAR2/FFAR3 activation are much higher than are clinically relevant, and more in the range of butyrate concentrations in the gut [339, 377, 379]. Human PBMCs from healthy donors were stimulated with monosodium urate crystals (to mimic acute gouty arthritis) and palmitate in the presence or absence of butyrate. The authors demonstrated a decreased production of IL-1β, IL-6, IL-8, and specific inhibition of class I HDACs when cells were treated with butyrate [379].

Many other *in vitro* studies report strong immune-modulating effects of butyrate by inhibiting production of pro-inflammatory cytokines and nitric oxide, and blocking NF-κB activation [380-384]. Some studies however show an increase in IL-10 upon butyrate stimulation. An *in vitro* study on human monocytes showed that sodium butyrate inhibited S.*aureus*-induced production of pro-inflammatory IL-12, by suppressing IL-12p35 and IL-12p40 mRNA accumulation, but enhanced IL-10 secretion [383]. In the present study however, PBMCs were used and not solely human monocytes. Even though frequencies vary among individuals, human PBMCs consist of 70-80% lymphocytes (of which 70-85% are T cells, 5-10% B cells and 5-20% NK cells), 10-20% monocytes and only 1-2% dendritic cells [385]. It is therefore expected that most effects seen in Figure 3-2 are T-cell-dependent effects. Butyrate potentiates DCs to convert naïve T cells into Tregs and suppresses their conversion into pro-inflammatory (IFN-γ$^+$) T cells [386]. This Treg promoting effect is mediated via activation of GPR109a [387]. Butyrate activation of GPR109a in macrophages and DCs is essential for maintaining the balance between pro- and anti-inflammatory CD4+ T cells. Butyrate also directly interacts with T cells, facilitating Treg polarisation *in vivo* and *in vitro*, possibly via HDAC inhibition [388, 389].

Butyrate (and acetate and propionate) directly promote T cell differentiation into IL-17, IFN-γ producing T cells, and/or IL-10 depending on the cytokine milieu. These effects depend on direct HDAC inhibitor activity. HDAC inhibition in T cells increased acetylation of kinases regulating the mTOR pathway required for Th17, Th1 and IL-

10^{+} T cells. The authors concluded that SCFAs promote T cell differentiation into both effector and regulatory T cells depending on the immunological milieu [390]. SCFAs also impair Th2 polarisation, which has important allergy reducing effects. These effects were dependent on FFAR3 but not FFAR2 [391]. Effects of SCFAs on other immune cells are reviewed in [234].

As noted previously, butyrate is a short-chain fatty acid produced by the gut microbiome, where it plays an important role in maintaining the mucosal barrier and regulating immune responses [392-394], reviewed by myself elsewhere [12]. Gut immune cells differ from blood mononuclear cells. Therefore, studying the effects of butyrate in an *in vitro* model of the gut may be of additional value.

3.6.2.3 Alpha-linolenic acid

Although n-3 PUFAs, and hence alpha-linolenic acid, are well-known for their anti-inflammatory effects, mixed effects were seen here (Figure 3-2). Indeed, alpha-linolenic acid decreased TNF secretion after both 4 hours and overnight stimulation, but had no effect (or even increased) IL-6 secretion after overnight stimulation. Furthermore, alpha-linolenic acid decreased secretion of the anti-inflammatory cytokine, IL-10.

IL-6 is generally considered to be pro-inflammatory when released chronically [395]. However, acute IL-6 release might be involved in recruitment of mononuclear cells [396] and an inducer of IL-10 synthesis [395], thereby exerting anti-inflammatory effects. An increase in IL-10 was not however shown here.

n-3 PUFAs are involved in reducing pro-inflammatory cytokine production, while increasing anti-inflammatory cytokines [170]. Here, only alpha-linolenic acid has been studied and not the fatty acids produced from alpha-linolenic acid. Potentially, alpha-linolenic acid itself is not the most potent n-3 PUFA, but more so EPA and DHA (the fatty acids also found in fish oils). EPA and DHA release resolvins and protectins, which control endogenous inflammation and immune responses [212, 397]. Many studies focus however on metabolic diseases such as diabetes, obesity or heart disease.

I did not perform any mRNA analyses; while this may have revealed a more in-depth analyses of the immunological effects of alpha-linolenic acid on PBMCs, mRNA levels are less clinically relevant than the protein.

As discussed previously, n-3 PUFA can also affect immune cell function by its incorporation into cell membranes. A higher n-6 PUFA/n-3 PUFA ratio was associated with a more anti-inflammatory immune function profile [398]. Cell membrane composition has not been studied here, but it could be a useful way to study the immunomodulating effects of alpha-linolenic acid other than cytokine secretion.

Many *in vitro* studies tend to use subclinical treatment doses of alpha-linolenic acid. In my study, I however chose to use clinically relevant concentrations which still may have been too low to impact on cytokine secretion.

3.6.3 Whole blood stimulation

When comparing whole blood results to PMBC stimulation, butyrate was not as anti-inflammatory. While it increased IL-1β secretion (and, correspondingly, inhibited IL-1RA), butyrate did not affect either TNF or IL-6 secretion.

Moreover, alpha-linolenic acid appeared more pro-inflammatory by increasing both IL-1β and secretion of IL-6 following co-intubation with LPS and *E. coli*. However, unlike PBMC stimulation, it did not affect IL-10 secretion. The same increasingly pro-inflammatory effect was seen for palmitate, although conflicting results were seen with IL-6 secretion with palmitate impairing LPS- and EC-induced secretion, but increasing *S. aureus*-induced secretion.

Both palmitate 0.4 mM and butyrate 1.8 mM reduced IL-10 production after overnight stimulation. This corresponds to their inhibitory effects of palmitate and on IL-10 secretion seen in PBMC supernatant (Figure 3-2).

Therefore, the effects of lipids seen in whole blood stimulation often did not correlate with what was found with PBMC stimulation. The concentration of immune cells is far lower in whole blood so cytokine effects might be less potent.

Also, whole blood contains clotting factors, neutrophils, reactive species and other factors involved in immunomodulation. These may have modulated the effects of the fatty acids. There may also be interactions between immune cell types. Neutrophil lifespan *in vitro* is relatively short and, on death, released damaging factors affect other cells. Furthermore, upon encountering microbes, neutrophils may form neutrophil extracellular traps (NETs), releasing their granules and producing inflammatory mediators including ROS [399], thereby leading to different cytokine profiles.

3.6.4   Flow cytometry

An extensive literature research was conducted on immune cells most affected in sepsis and how functionality could be investigated. Four immune cell types were chosen for further studies: T, B, dendritic cells and monocytes. Multicoloured flow cytometry panels were carefully designed and tested. Due to the high-cost and the low impact shown after two replicates at my host lab in Lausanne, this experiment was not followed up. These experiment methodologies are however of high value for future sepsis-related studies on these cell types. As these flow cytometry experiments were only been performed twice, the results described in section 3.5 are not discussed here in detail as it is potentially misleading to draw conclusions from such few studies.

In retrospect, it may have been worth focussing on lymphocytes (and potentially monocytes) rather than all different cell types, as these two cell types constitute 70-80% of all PBMCs. As greater effects were seen after overnight incubation, future studies should focus on this timepoint rather than 4 hour stimulation studies. Furthermore, the number or concentrations of fatty acids could be reduced. Based on cytokine secretion, examining palmitate at 0.4 mM and butyrate at 1.8 mM would be good options. Reducing the number of conditions, would allow more replicates. These experiments were performed at Lausanne University Hospital on a flow cytometer different from those at UCL and hence could not be reliably repeated.

From these experiments I can show that the methodology worked. A higher dose of *S. aureus* may have stimulated intracellular cytokine production better, though intracellular cytokine production is generally hard to measure as only the last few hours of production can be captured due to the toxicity of brefeldin A that needs to be added to prevent secretion of cytokines. For future studies, it may be worth examining total rather than intracellular cytokines, so no brefeldin A would have to be added. The supernatant can thus be kept for other analyses. It would also reduce the costs of the panels or make room for other (surface) antibodies to be added. For example, mitochondrial markers could have been of interest here. Mitochondrial markers were however used in the *in vivo* model of butyrate discussed in Chapter 4.

3.6.5 Conclusion

Palmitate and butyrate could both positively affect sepsis pathology, but in different phases. Butyrate was anti-inflammatory and could impact upon the pro-inflammatory phase of sepsis. Later in sepsis, in the immunosuppressed state, the pro-inflammatory effect of palmitate may be more useful though its cytotoxic effects would need to be carefully monitored.

For future work, I would reduce the number of conditions for flow cytometry experiments to look at T cells (and monocytes) specifically. I would also add mitochondrial markers to these studies. Additionally, I would look at underlying mechanisms (such as NF-κB, TLR, GPR and HDAC expression) involved in effects on cytokine secretion shown here.

Chapter 4   The impact of fatty acids on mitochondrial function in an *in vitro* model of sepsis

4.1   Introduction

Mitochondrial dysfunction is a hallmark in sepsis, as described in section 1.5. Fatty acids feed electrons into the respiratory chain and hence are excellent respiratory substrates. Depending on chain length, degree of saturation, and concentration, fatty acids differently affect mitochondrial respiration [297]. Other than its role as a substrate, lipids can impact on mitochondrial function through their uncoupling effect, as fatty acids act as protonophores and affect genes involved in uncoupling. The mitochondrial stress test was performed to study the impact of palmitate, butyrate and alpha-linolenic acid on mitochondrial respiration as described in section 2.6.

4.2   Results

Oxygen consumption rates are shown in Figure 4-1. None of the parameters showed statistically significant different results compared to control, despite six separate studies, due to wide error bars. However, when analysing butyrate only, butyrate at the 1.8 mM concentration does have an increased uncoupling effect in the *S. aureus*-treated groups. This is shown by increased spare capacity and maximal respiration (data not shown). I here however chose to show all tested conditions and acknowledge low sample sizes and a less powerful test with a possible type II error (where sample sizes are too small to show a difference).
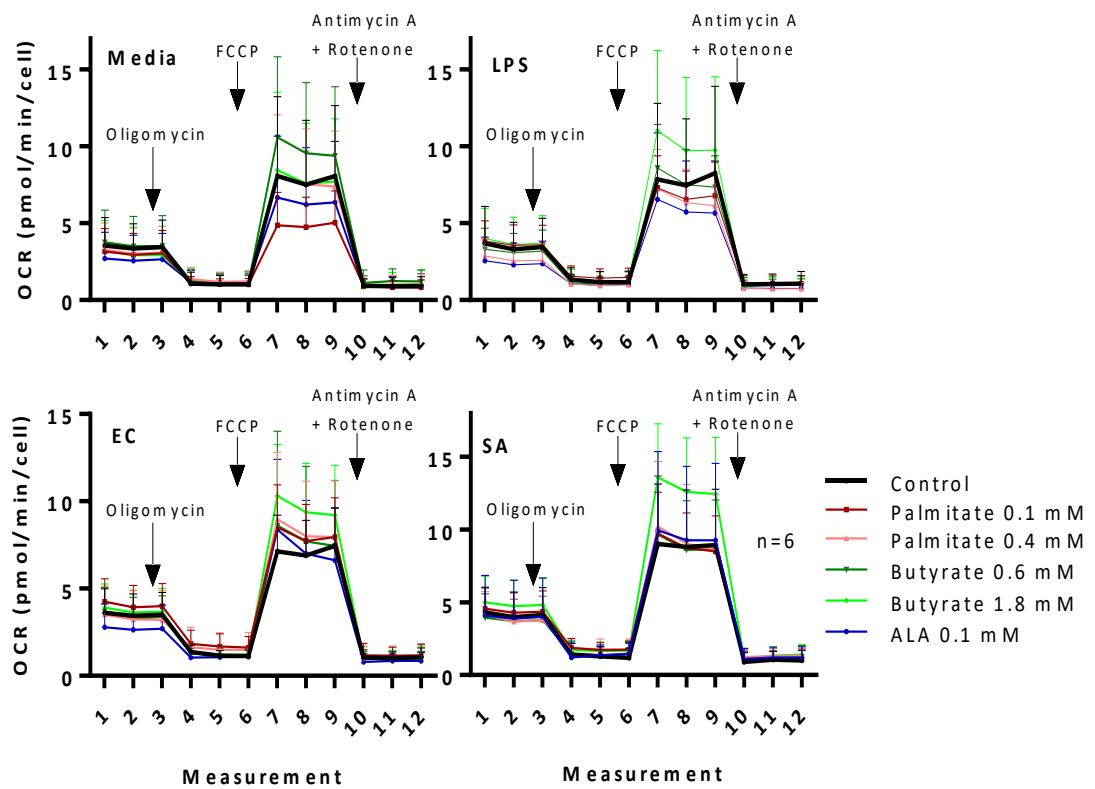
Figure 4-1: Oxygen consumption rates of human PBMCs stimulated overnight and treated with different fatty acids (n=6). Results given as mean+SD.

Figure 4-2 represents changes as percentage change from control, allowing better visualisation of the effects on the different components of respiration. Palmitate at 0.1 mM non-significantly increased basal respiration, predominantly due to a non-significant increase in proton leak, under all conditions. Maximal respiration and spare respiratory capacity were unaffected. On the other hand, butyrate had no effect on ATP production, basal respiration or proton leak but maximal respiration and spare respiratory capacity were non-significantly increased under all conditions. Alpha-linolenic acid at a concentration of 0.3 mM significantly diminished mitochondrial respiration (data not shown), likely related to toxicity effects. However, at the lower concentration (0.1 mM) alpha-linolenic acid non-significantly impaired basal and ATP-linked respiration, and increased proton leak.
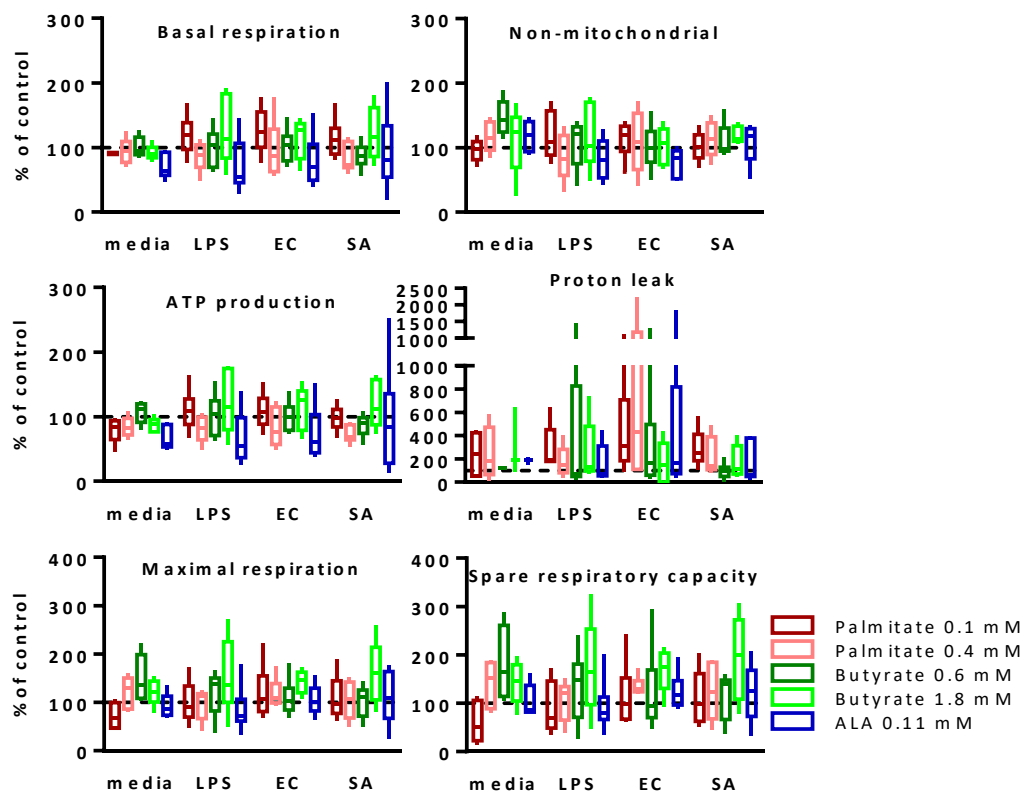
Figure 4-2: Mitochondrial respiratory variables of human PBMCs stimulated overnight and treated with fatty acids (n=6). Presented as percentage of control (mean±SD), i.e. stimulus without fatty acids.

4.3 Discussion

Maximal respiration and spare respiratory capacity were only significantly increased in *S. aureus*-stimulated PBMCs exposed to 1.8 mM butyrate. No overall statistical significance was seen in this overnight stimulation study despite testing six samples per group and condition as the inter-sample variability was too high. Low sample size with high variance might have caused a type II error, where non-statistically relevant results are found even if there is a difference. I chose to use a non-parametric test, as these are biological samples (which usually have high variability) and I only had a low sample size, to avoid so-called 'p-hacking'. Furthermore, a statistically significant difference does not necessarily mean that it is a biologically important result. Moreover, a non-statistically significant result does not necessarily mean it is scientifically irrelevant. This is especially the problem with small sample sizes. The interpretation of the results discussed below hence is not supported by strong statistical evidence, but may still be scientifically relevant and worth consideration. For future studies and to draw better statistical conclusions, I would focus on butyrate and increase sample sizes.

4.3.1 Palmitate

As a long-chain fatty acid palmitate would be expected to have potent uncoupling effects, increasing proton leak and thus maximal respiration and spare capacity by increasing proton conductance. Here, protons 'leak' back across the mitochondrial inner membrane, diminishing membrane potential and increasing activity of the respiratory chain to try to maintain the potential [61, 400]. This was the signal detected at both concentrations and under all conditions (control and pathogen-stimulated).

Proton leak-linked respiration is highly dependent on the protonmotive force that drives it. Changes in protonmotive force alter proton leak-linked respiration without changing proton conductance of the inner membrane [401]. In my studies, proton leak-dependent respiration was non-significantly increased with maximal respiration (uncoupler induced) unaffected. This suggests a difference in membrane

conductance as a change in membrane potential does not explain these results [400] [64].

These results also differ from those obtained in a study on cultured myotubes from ICU patients [355]. While an increase in basal respiration was also seen, fatty acid oxidation capacity was 157% higher than controls with a significant increase seen in maximum respiratory chain capacity. Even in the presence of glucose and insulin, elevation of FFA in the extracellular environment increased maximal respiratory capacity, suggesting an increase in uncoupling.

The non-signficant increase in proton leak-associated respiration in palmitate-treated PBMCs can reflect mitochondrial damage [402] or a physiological response [64]. Palmitate induced mitochondrial DNA damage in myoblasts, and this was associated with reduced basal, ATP-linked and maximal mitochondrial respiration [353].

Both ANT and UCPs play a role in proton leak, as described in section 1.10. Several studies suggest a link between FFAs and UCP-1 activity [58]. Palmitate can physically interact with UCP-1, leading to a conformational change that induces mitochondrial uncoupling [403]. Palmitate may also increase proton conductance. Binding of LCFA (palmitate or oleate) to the ANT resulted in fatty acid-facilitated proton conductance through this translocator [301]. This protonophore activity was confirmed by an increased rate of dissipation of mitochondrial membrane potential yet a parallel stimulation of resting respiration. Interaction between LCFA and ANT may induce mitochondrial swelling via opening of the PTP (permeability transition pore) [297].

Most studies have looked at skeletal muscle cells and not PBMCs. Palmitate may have different effects on different cell types. Even though I saw a non-significant uncoupling effect as shown by non-significant increased proton leak, I did not identify specific mechanisms. Future studies could look at alterations in UCP and ANT expression and protein levels and measurement of mitochondrial membrane potential.

An increase in proton leak may suggest increased mitochondrial uncoupling. Mitochondrial uncoupling is often viewed as dysfunctional, but it has functional effects that are relevant in infection. Uncoupling produces heat, which may help to fight infection by direct bactericidal effects [335]. Uncoupling also affects immune cells [336] and induces heat shock proteins [337]. Reduced ROS production through lowering of mitochondrial membrane potential may prevent exaggerated tissue damage in sepsis.

I did not measure mitochondrial membrane potential nor ROS production in these studies as the cell numbers required for Seahorse experiments was high. I did later examine these by flow cytometry in the *in vivo* butyrate infusion study (Chapter 5).

4.3.2 Butyrate

Butyrate induces mitochondrial respiration and fatty acid oxidation both *in vitro* and in murine studies [404-406]. Here, butyrate non-significantly increased both maximal respiration and spare respiratory capacity in the present study in a dose-dependent manner (Figure 4-1).

Models assessing the effects of butyrate on mitochondrial respiration vary significantly. There are differences in fatty acid administration, cells studied and doses used. It is increasingly clear that there is a marked variation in metabolism between cell types [401]. This study therefore adds respirometry data, albeit specifically for PBMCs.

The literature using comparable study designs is scarce. One model used starved human endothelial cells and monocytes exposed to hexanoic acid (C6), heptanoic acid (C7), octanoic acid (C8) or glucose while inflammation was mimicked by adding TNF [407]. MCFAs and SCFAs increased mitochondrial respiratory capacity both at baseline and under inflammatory conditions. None of the fatty acids changed mitochondrial DNA content nor generation of proinflammatory cytokines. In monocytes, TNF significantly reduced maximal respiration, but this could be reversed by incubation with glucose and fatty acids. Octanoic acid exerted the highest increase in maximal respiratory capacity.

I however found no clear difference in basal and maximal respiration between pathogen-stimulated and unstimulated PBMCs (Figure 4-1). Butyrate did non-significantly increase basal respiration, more so in pathogen-stimulated groups. This can be attributed to a non-significant increase in both ATP-linked respiration and proton leak as shown in Figure 4-2. Butyrate hence might positively affect maximal respiration and spare capacity and hence makes PBMCs more able to respond to stress caused by sepsis. As ATP-linked respiration was also non-significantly enhanced, taken together butyrate may be a more useful fatty acid as a sepsis therapeutic compared to palmitate. As described earlier with palmitate, I did not assess the impact of butyrate on mitochondrial membrane potential nor ROS production in this model but this was done in a subsequent model.

4.3.3 Alpha-linolenic acid

As discussed in section 1.10, the uncoupling potency of fatty acids generally increases as chain length and unsaturation increase [297, 300, 301]. Based on this premise, alpha-linolenic acid would have the highest uncoupling effect. This fatty acid has a longer chain length than palmitate or butyrate, and is unsaturated whereas palmitate and butyrate are both saturated.

Alpha-linolenic acid non-significantly increased spare capacity after stimulation with EC and SA, but not LPS. Spare capacity represents the difference between basal and maximal respiration. As Figure 4-2 shows a non-significant decrease in basal respiration, an increase in spare capacity could be expected. The non-significant decrease in ATP-linked respiration may also be linked to the decrease in basal respiration. However, it could also be a result of decreased cellular ATP demand or a reduced ability to synthesise ATP (by impaired ATP synthase activity or dysfunction of the ANT) [401]. It is perhaps most likely related to a reduced ability to generate a mitochondrial membrane potential to drive the ATP synthesis rate, as shown by a reduction in maximal respiration (Figure 4-2). Measuring membrane potential would have been of additional value here.

Most research on n-3 fatty acids in respect to mitochondrial function has been focussed on EPA and DHA, and not alpha-linolenic acid. As described before, alpha-

linolenic acid can endogenously be converted into EPA and DHA, but tracer studies demonstrated a low conversion efficiency (<8%) [408, 409]. In a study using obese mice, alpha-linolenic acid supplementation increased maximal respiration in skeletal muscle [410]. n-3 fatty acids are known to affect mitochondrial membrane composition [411, 412]. This may potentially affect mitochondrial respiration although no effect on maximal respiration was seen in skeletal muscle cells taken from healthy subjects exposed to a n-3 fatty acid diet [412].

Skeletal muscle cells differ from PBMCs. This potentially explains the difference in findings. Alpha-linolenic acid at a concentration of 0.3 mM diminished mitochondrial respiration, and higher doses had a greater effect. This alpha-linolenic acid dose may be causing toxicity as suggested by MTT, tryphan blue, and flow cytometry.

4.4   Conclusion

Although no statistically significant results were obtained with six replicates comparing all three fatty acids together, butyrate seems to be the most promising fatty acid to use as a sepsis therapeutic. Butyrate non-significantly increased maximal respiration and ATP-linked respiration, which may especially impact upon the hypometabolic phase in sepsis. Palmitate could be of use in the early pro-inflammatory phase of sepsis considering also its physiological effects of proton leak and uncoupling, although it may potentially damage mitochondria. Alpha-linoleic acid appeared to induce toxicity. Thus I took forward a butyrate infusion to test in a long term *in vivo* rat model of sepsis.

For future experiments, sample size should be increased and flow cytometric analyses of mitochondrial function using dyes for mitochondrial membrane potential and mitochondrial ROS production could be added. This could have been combined with the panels already made for immune function, but in a simplified version. Furthermore, ANT and UCP expression analyses might give mechanistic insight in how fatty acids, and here palmitate, increases uncoupling. There are links between LCFA and ANT. UCP-2 is would be most relevant, as it is expressed on

immune cells and is involved in cell activation, mitochondrial ROS production and fatty acid oxidation [336].

Of note, these experiments were however performed at UCL, where, unlike in Lausanne, there was no availability of buffy coats from a blood bank. Blood was therefore withdrawn from healthy volunteers at UCL and hence lower cell numbers were obtained. Although same culture conditions used in immune function studies were used to study mitochondrial respiration, a maximum of 300,000 cells per well could obtained, instead of 1,000,000 cells per well used in Lausanne. For Seahorse purposes however, between 100,000 to 500,000 PBMCs are normally used to allow changes in oxygen consumption upon drug injections. At the end of incubation, cells were resuspended in Seahorse medium with no fatty acids and processing usually took about an hour before the test was started. This could affect mitochondrial respiration, though impact is expected to be minimal.

Chapter 5   Impact of butyrate infusion in an *in vivo* model of sepsis

5.1   Introduction

Although *in vitro* studies are generally rapid and allow high throughput, they do not give insight into what happens on a whole-body level with organ interaction. Based on the previously discussed *in vitro* studies on immune function and mitochondrial respiration, butyrate proved most promising as a sepsis therapeutic. An *in vivo* model of sepsis was designed to study the effects of a butyrate infusion on a whole-body level. I used a rat model of rat faecal peritonitis that is well established within the lab and before commencing butyrate studies sought to characterise it in my hands.

5.2   Characterisation study

5.2.1   Faecal slurry dose finding and clinical severity scoring

Using the microbial assessment performed in 2018 by Dr. Sean Pollen in the host lab, the faecal slurry I used contained 1.4×10^4 viable aerobic and 1.8×10^6 viable anaerobic bacteria. Rats initially received a faecal slurry dose of 4 µl/g body weight and all survived to 72h. Their clinical severity score never exceeded 1, although evidence of a generalised inflammatory peritonitis response (purulent intraperitoneal fluid excess, abscess formation etc.) was visually identified. Mortality rate was 22% on increasing the faecal slurry dose to 5 µl/g; two out of twelve animals were culled for distress. Post-mortem findings were similar to the lower dose of 4 µl/g. The 72-hour mortality rate increased to 33% (five out of fifteen) when the dose was increased to 6 µl/g, with two unexpected deaths overnight and three culled due to prolonged laboured breathing. All animals (four out of four) were culled due to distress when faecal slurry dose was raised to 7 µl/g as their breathing was very laboured shortly after sepsis induction (Figure 5-1).
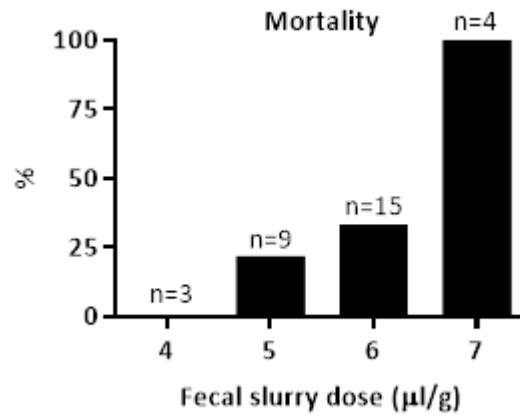
Figure 5-1: 72-hour mortality rates observed with different doses of intraperitoneal faecal slurry injections.

5.2.2 Echocardiography and core temperature

As shown in Figure 4-2, stroke volume and cardiac output at 6 hours fell significantly in the septic animals compared to sham controls (sham operated animals by Miranda Melis), but though the values were trending lower there was no significant difference between eventual survivors and non-survivors. Similarly, core temperature fell in the septic animals, more so for eventual septic non-survivors but this was not significantly different. Of note, these measures were taken in anaesthetised animals and hence may not accurately reflect human sepsis, as discussed later.
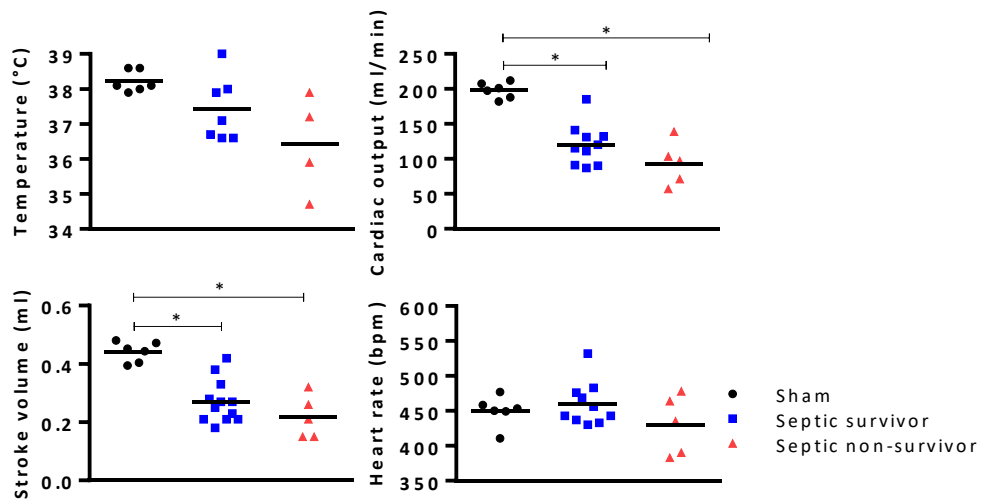
Figure 5-2: Cardiorespiratory variables measured 6 hours after sepsis induction for sham animals, septic survivors and septic non-survivors. Bar at mean. *p<0.05

5.2.3 Metabolic monitoring

VO_2 levels (Figure 5-3) were lower in septic versus sham-operated animals. VO_2 consumption was non-significantly lower in septic non-survivors compared to septic survivors. One animal deteriorated before it was culled due to distress.
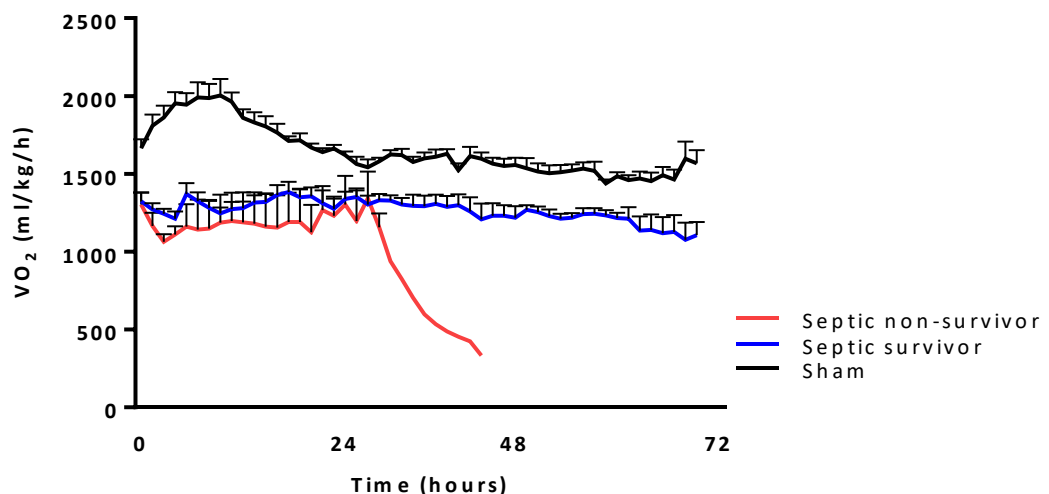
Figure 5-3: Whole body oxygen consumption (VO_2) for sham animals (n=6), septic survivors (n=10) and septic non-survivors (n=5) (mean+SD). *p<0.05 sham vs septic survivor and sham vs septic non-survivors.

The VCO_2 profile (Figure 5-4) was similar to the VO_2 profile. Septic animals had a lower VCO_2 production than sham animals, and levels in septic non-survivors were non-significantly lower compared to septic survivors. A drop in VCO_2 levels was seen in one animal that deteriorated before it was culled due to distress.
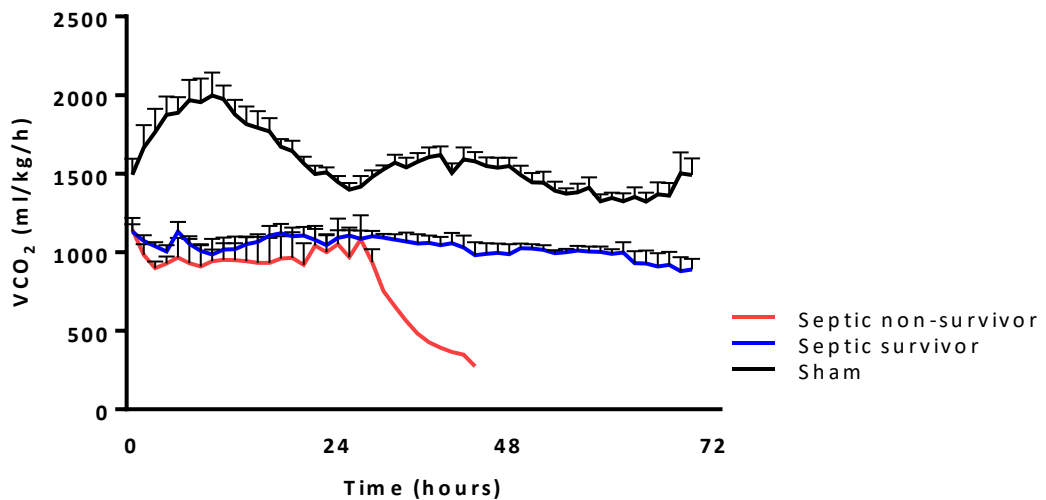
Figure 5-4: Whole body carbon dioxide production (VCO_2) for sham animals (n=6), septic survivors (n=10) and septic non-survivors (n=5) (mean+SD). *p<0.05 sham vs septic survivor and sham vs septic non-survivors.

Soon after sepsis induction, a decrease in respiratory exchange ratio (RER) was observed (Figure 5-5). The RER of sham rats dropped early after surgery, but promptly increased and stayed between 0.9-1.0 thereafter, indicative of predominant carbohydrate metabolism. For septic animals receiving a slurry dose of 6 μl/g, the RER fell to approximately 0.8, indicating predominant utilisation of protein and fat. There was no difference in RER between eventual survivors and non-survivors.

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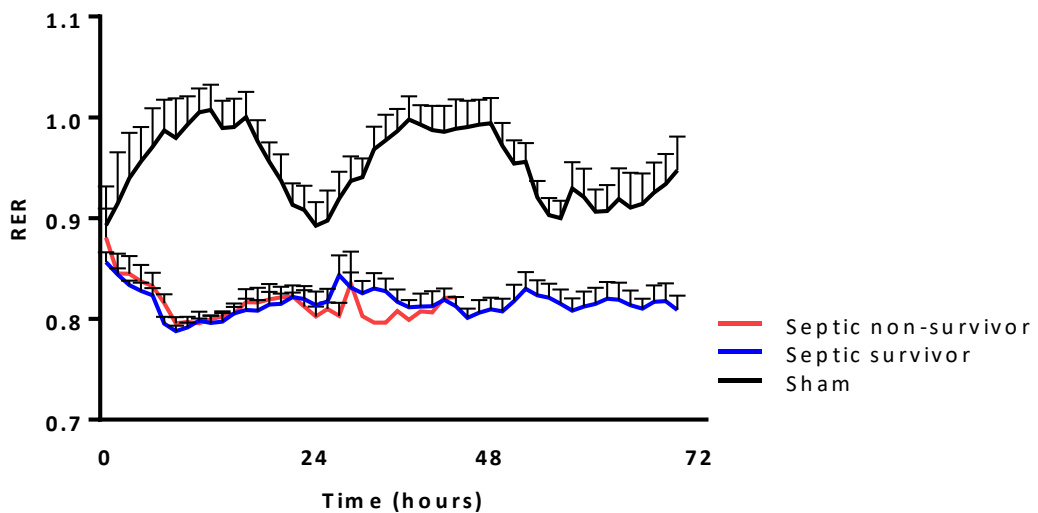
Figure 5-5: Respiratory exchange ratio (RER) for sham rats (n=6), septic survivors (n=10) and septic non-survivors (n=5) receiving a faecal slurry dose of 6 µl/g. Mean+SD. *p<0.05 sham vs septic survivor and sham vs septic non-survivor.

Of note, the RER fluctuated over the day in the sham animals, corresponding with sleep/awake patterns, but this diurnal variability was lost in the septic animals and had still not returned to normal after clinical recovery at 72 hours.

5.3 Safety study

Based on the results of my *in vitro* studies of fatty acids on immune and mitochondrial function, I chose to investigate the impact of butyrate in *in vivo* studies. Butyrate at a concentration of 1.8 mM had most impact on both immune and mitochondrial function, and I therefore aimed to reach this concentration in the circulation. I was unable to measure plasma butyrate levels but pharmacokinetic studies showed that a continuous infusion of 0.6 g/kg/h of sodium butyrate would reach a plasma concentration of 1.8 mM [413, 414].

Safety was initially assessed in the anaesthetised rat by stepwise increase of sodium butyrate doses. A 10-fold intended drug dose was difficult to dissolve, and the animal died while this was being infused. Animals were however stable at lower doses (0.1, 0.3, 1 and 3 times intended drug dose).

In another safety study, the intended drug dose of 0.6 g/kg/h was infused and the animal monitored for 90 minutes before the dose was repeated. There were no obvious issues and the 90 min end point of this study was successfully reached.

Safety was then assessed using a butyrate infusion model using healthy awake, instrumented animals in metabolic cages. A drop in RER was seen directly after starting butyrate infusion and further decreased as the infusion rate increased. This indicates a switch towards fat metabolism. The RER rose when the butyrate infusion was reduced. The RER of control animals remained stable at 0.9-1.0 (Figure 5-6).
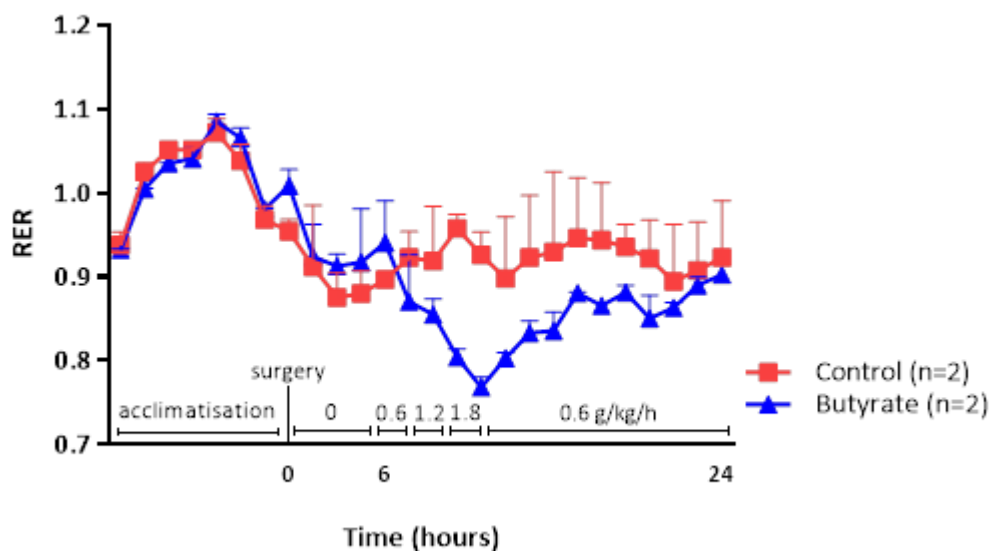
Figure 5-6: Respiratory exchange ratio (RER) for butyrate-infused healthy rats and control animals in a safety study (n=2). Mean+SD.

Table 5-1 shows blood gas, echo and temperature data in awake animals treated with butyrate or control fluid. Temperature was reduced in the butyrate-treated animals, especially at study endpoint. pH, base excess and bicarbonate (HCO_3^-) were higher in the butyrate-treated animals indicative of a metabolic alkalosis. There was hypercapnia from a compensatory respiratory acidosis. Glucose and lactate levels were also elevated in butyrate-treated animals. Despite these findings the animals appeared normal so I decided to proceed to infuse 0.6 g/kg/h butyrate to septic animals.

Table 5-1: Mean blood gas and echo values for safety study in awake animals 24 hours after surgery (n=2).

Treatment	Control	Butyrate
Temperature (°C)	37.5	35.7
pH	7.41	7.50
$PaCO_2$	6.67	7.78
K^+ (mmol/l)	4.3	3.4
Na^+ (mmol/l)	143	149
Ca^{2+} (mmol/l)	1.01	0.99
Cl^- (mmol/l)	101	92
Glucose (mmol/l)	9.2	13.9
Lactate (mmol/l)	2.2	3.4
BE (mmol/l)	7.3	22.5
HCO_3^- (mmol/l)	27.4	40.8
Cardiac output (ml/min)	177	162
Stroke volume (ml)	0.39	0.41
HR (beats/min)	451	393

5.4 Butyrate infusion study

5.4.1 Clinical severity scoring

Naïve animals all scored 0 on the clinical severity score. There was no difference in clinical severity and weight loss between septic control and septic butyrate-infused animals. Septic animals did not exceed a severity score of 1, and hence none were culled before the desired endpoint.

5.4.2 Metabolic monitoring

VO_2 levels of septic animals were non-significantly lower compared to naïve animals (Figure 5-7). Naïve animals did not undergo surgery and received no i.p. injection. There was no difference in VO_2 consumption between septic control and septic butyrate-infused animals.
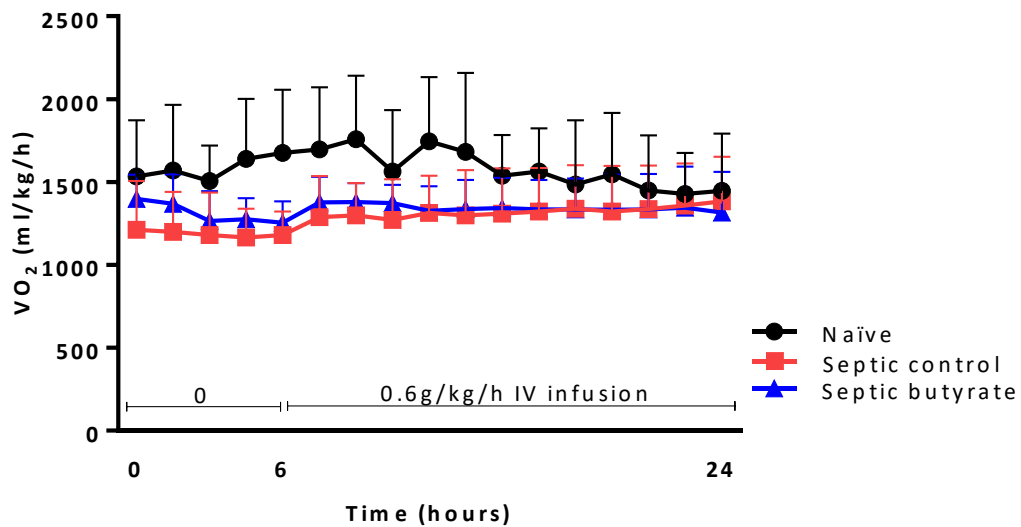
Figure 5-7: Whole body oxygen consumption (VO$_2$) of naïve animals (n=4), septic control (n=8) and septic butyrate (n=8). Mean+SD.

The VCO$_2$ profile (Figure 5-8) showed a similar trend compared to the VO$_2$ profile.
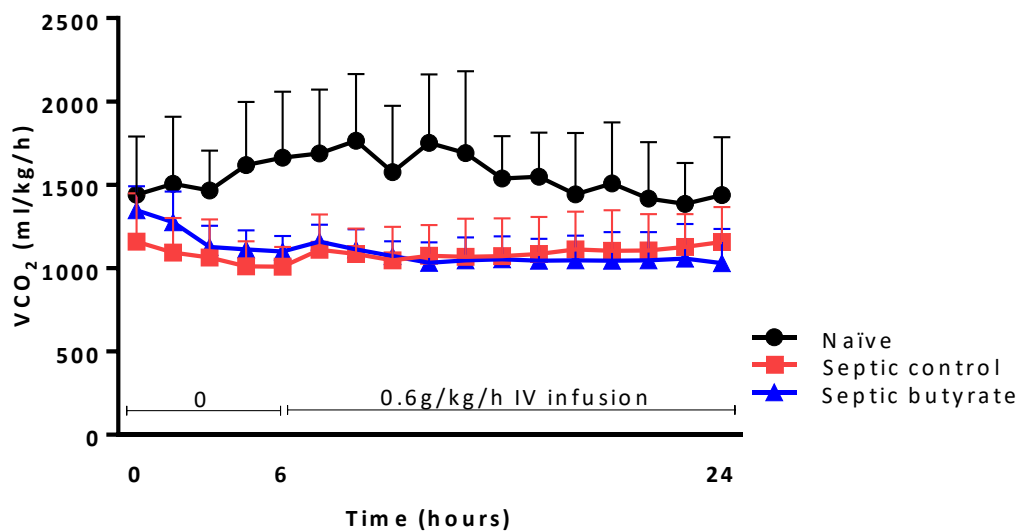
Figure 5-8: Whole body carbon dioxide production (VCO$_2$) of naïve animals (n=4), septic control (n=8) and septic butyrate (n=8). Mean+SD.

Septic animals, both butyrate-treated and non-treated had a significantly lower respiratory exchange ratio compared to naïve animals. The RER of septic control and septic butyrate-infused animals was similar both before and after commencement of the butyrate infusion at 6h. The butyrate-infused septic animals

had a non-significantly lower RER compared to septic control animals (Figure 5-9). RER is based on the formula: RER = VCO_2 / VO_2.
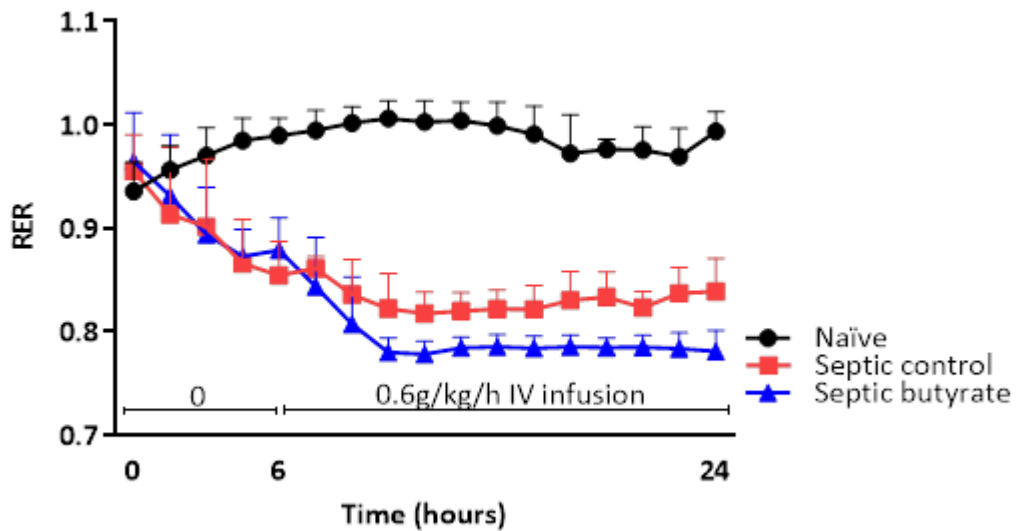
Figure 5-9: Respiratory exchange ratio of naïve animals (n=4), septic control (n=8) and septic butyrate (n=8). Mean+SD. *p<0.05 naïve – septic control and naïve – septic butyrate.

5.4.3   Echocardiography and blood gases

Blood gas and echo values for naïve, septic control and septic butyrate infused animals are shown in Figure 5-10. Temperature, chloride concentration and cardiac output in septic butyrate infused animals were significantly lower compared to septic controls. Arterial pH, base excess, HCO_3^- and Na^+ concentrations in these animals were significantly higher compared to both naïve and septic control animals, whereas K^+ concentrations were significantly lower. Glucose levels fell significantly in septic controls, but were similar to naïve animals in the butyrate infused group. Lactate concentration was significantly higher in the butyrate infused group.

Septic butyrate-infused animals had a significantly lower stroke volume compared to naïve rats, but not to septic control rats. Whereas heart rate was higher in the septic control group compared to naïve animals, the butyrate-infused septic animals had a heart rate similar to the naïve group.

The butyrate-treated septic animals also had a lower temperature, potassium and chloride and a higher arterial pH, sodium and lactate levels compared to non-septic controls. The blood glucose in the butyrate group was similar to sham controls but significantly higher than the septic controls.
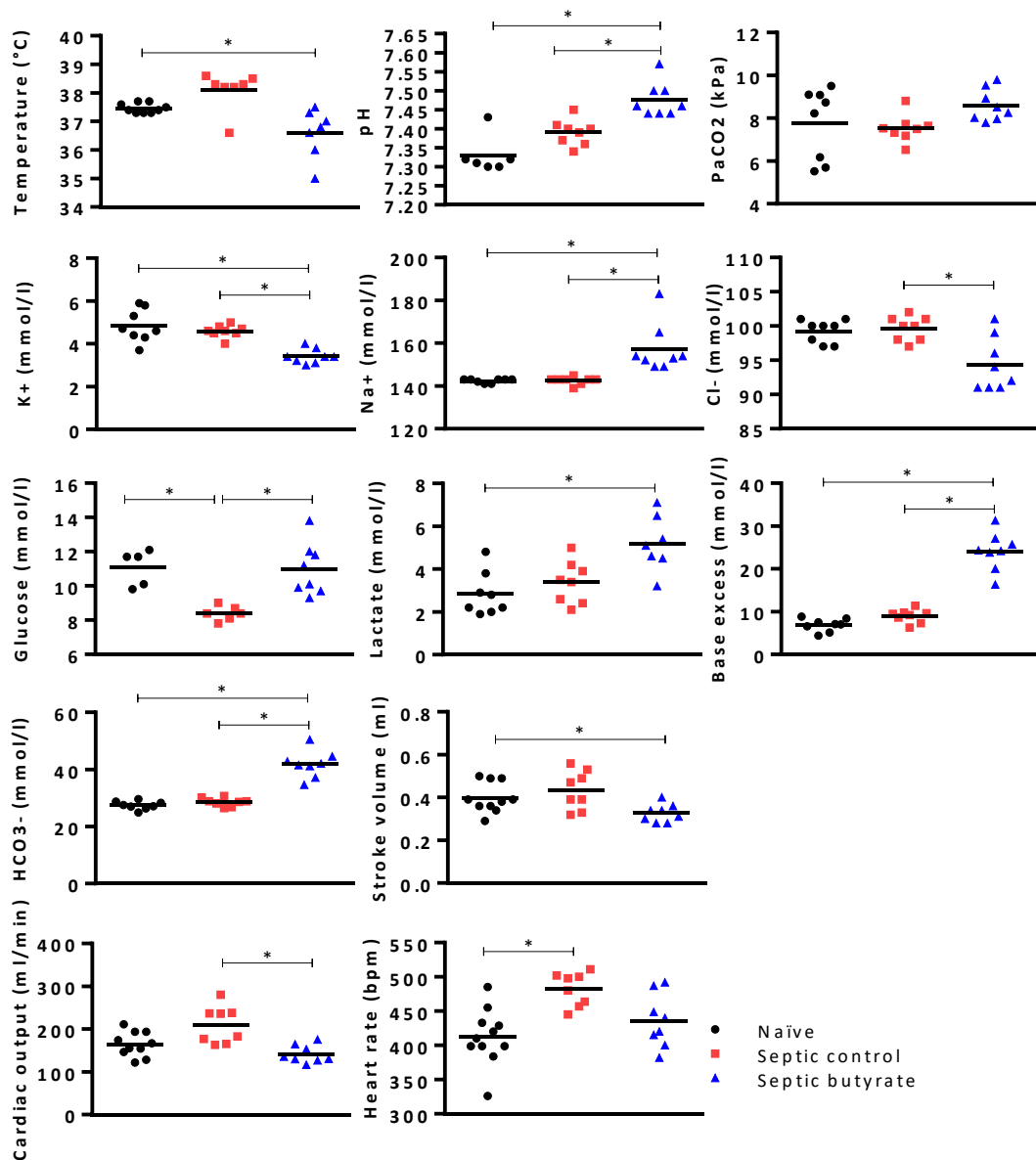
Figure 5-10: Blood gas, echocardiographic and metabolic values for naïve, septic control and septic butyrate infused animals. Bar at mean. *p<0.05.

5.4.4 Mitochondrial respiration of splenocytes

Splenocytes isolated from septic rats show an impaired rise in oxygen consumption rate (OCR) after addition of the uncoupling agent, FCCP, as compared to naïve rats (Figure 5-11). Splenocytes from septic rats receiving an overnight butyrate infusion commencing at 6 hours had further impairment of OCR compared to both naïve and septic control animals. Individual respiratory parameters are shown in Figure 5-12.

Basal respiration in splenocytes from butyrate-infused septic animals was significantly lower compared to naïve rats. Both butyrate-treated and non-treated septic splenocyte groups had significantly reduced maximal respiration and spare capacity with a further decrease in maximum respiration in the butyrate group. Non-mitochondrial respiration was significantly reduced in the butyrate-infused group compared to naïve, but not against splenocytes from septic controls. This was also found for ATP-linked respiration. A non-significant increase in proton-leak linked respiration was found in splenocytes from septic control animals, though proton leak was significantly reduced in those taken from septic butyrate-treated animals.
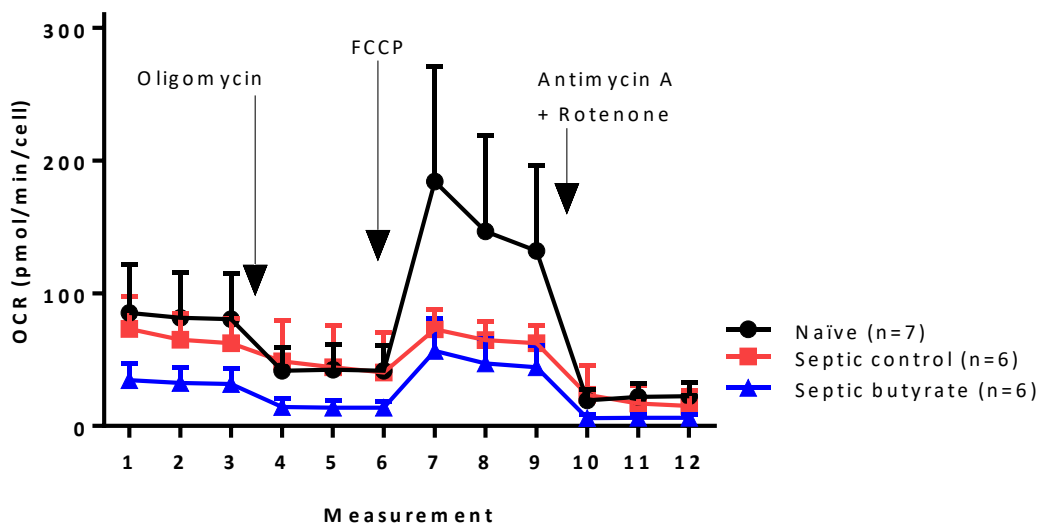
Figure 5-11: Oxygen consumption rate of splenocytes isolated from rats. Data shown as median and interquartile range. Mean+SD.
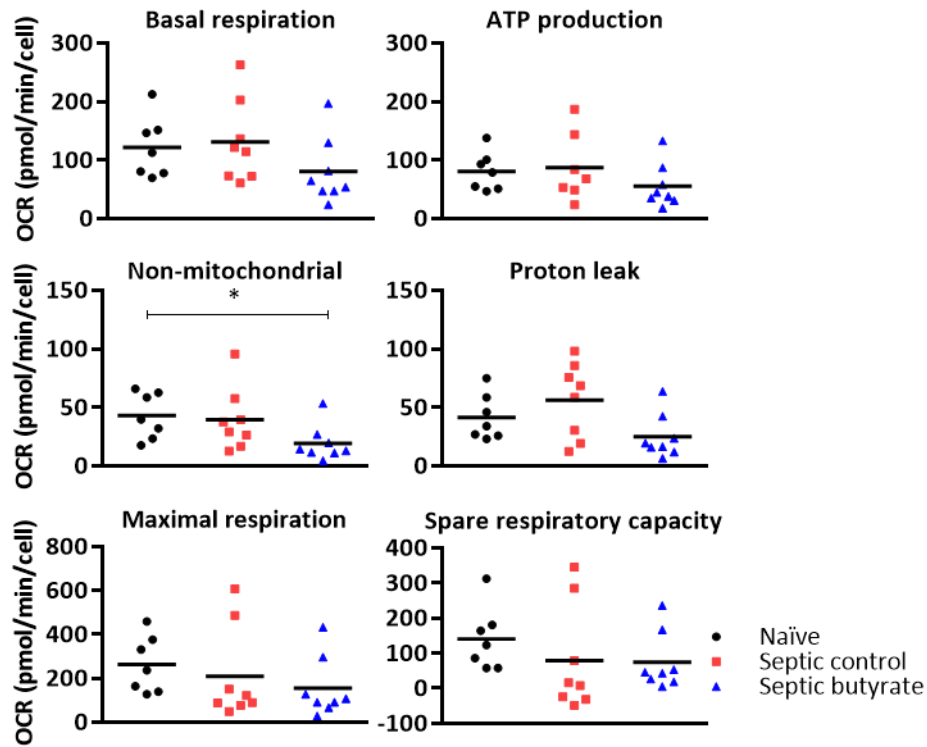
Figure 5-12: Mitochondrial respiratory variables of rat splenocytes. Bar at mean. *p<0.05.

5.4.5 Flow cytometry splenocytes

Flow cytometry results are shown in Figure 5-13. Results are shown for all isolated splenocytes as the sample size is bigger. CD4+ T helper cells show a similar trend to isolated splenocytes (data not shown). There were non-significant increases in IL-10 production in splenocytes isolated from septic rats, higher in those from butyrate-infused animals. Splenocytes from septic rats produced more TNF as opposed to naïve animals (p<0.05), but this was unaffected by butyrate treatment. Splenocytes from septic rats infused with butyrate had a significantly higher membrane potential and, consequently, a significantly higher mitochondrial ROS production (about 10-fold). Sepsis alone only caused a small non-significant increase in mitochondrial ROS production.
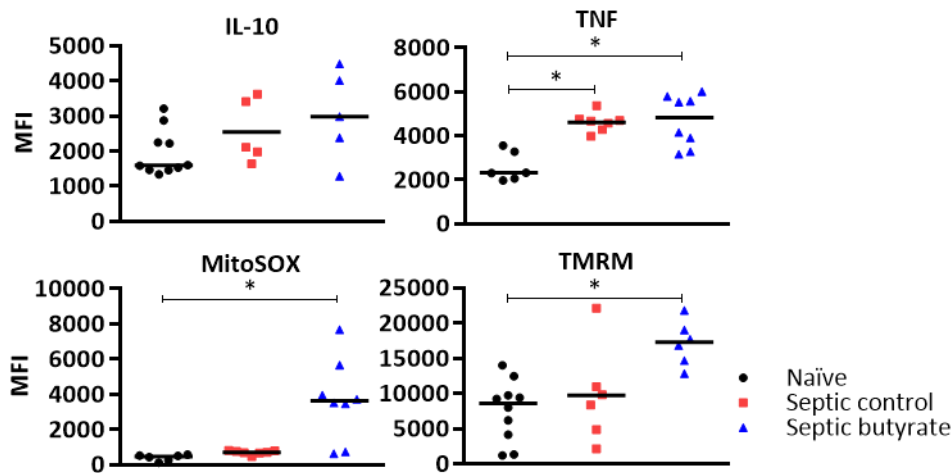
Figure 5-13: Mitochondrial membrane potential, cytokine and mitochondrial ROS production by rat splenocytes. Bar at mean. *p<0.05.

5.4.6 LPS and PMA stimulation

Freshly isolated rat splenocytes (from naïve, septic control and septic butyrate animals) were stimulated overnight with LPS (100 and 1000 ng/ml) and phorbol 12-myristate 13-acetate (PMA, 200 nM), after which supernatant was collected for cytokine measurements. Very low or no cytokines could be measured in either plasma and supernatant of stimulated splenocytes.

5.5 Discussion

5.5.1 Characterisation study

I initially aimed to identify the dose of faecal slurry insult that would generate a desired mortality of approximately 40% at 72 hours. This reflects the approximate mortality rate in patients suffering from faecal peritonitis. Unlike human patients, this animal model gets neither source control of the sepsis (in this case, peritoneal washout) nor antibiotic administration. Septic patients also frequently receive other immunomodulatory drugs such as sedatives, catecholamines and corticosteroids. So, other than fluid resuscitation and analgesia, this follows a more natural course of events of a septic insult.

This animal model aims to represent a clinically relevant model of critical illness. As seen in patients undergoing emergency laparotomy for faecal peritonitis, post-mortem examination of the rat peritoneum showed abscess formations plus numerous adhesions and oedematous loops of bowel [415]. In this way, faecal peritonitis could be visually confirmed. However, whereas over 70% of patients with faecal peritonitis have numerous comorbidities such as diabetes, cardiovascular disease and renal dysfunction [416], and are usually older, the animals are young and healthy before onset of the disease. Also, unlike patients, the rats only received fluid resuscitation and no antibiotics, catecholamines or mechanical ventilation.

The faecal slurry dose of 6 µl/g is similar to that used by other researchers in the lab. Animals surviving beyond 48 hours show clear signs of clinical recovery at 72 hours, with increased activity and an improved appearance. Normal diurnal rhythm, as reflected by RER, was however not restored after clinical recovery. Six hours' post-sepsis induction reflects an early phase of sepsis. Sepsis is established by 24 hours and surviving animals are in the recovery phase at 72 hours. The majority of animals dying do so between 18-40 hours, so most will still be alive at 24 hours after sepsis induction. I thus selected this timepoint as the desired endpoint for the butyrate infusion study.

Echocardiography was performed under brief isoflurane anaesthesia 6 hours after sepsis induction to assess cardiac function and validate the model. Despite large volume fluid resuscitation there was a significant fall in stroke volume and cardiac output in septic animals. Vasoconstrictive mediators like catecholamines are widely used in human sepsis and septic shock. In septic shock, with persisting hypotension, vasoplegia (low systemic vascular resistance with normal or raised cardiac output) is commonly due to persistent and irreversible hypotension. The latter is associated with vascular hyporeactivity to vasoconstrictors [417]. Although current model may thus not represent human (late) sepsis and no catecholamines were used, the fall in stroke volume and cardiac output in septic animals is likely due to myocardial depression as hypovolaemia had been largely corrected [418]. The decrease in cardiac output may also relate to the use of anaesthetics when taking the measures.

Of note, the use of anaesthetics here may explain why the model not accurately reflects (non-anaesthetised) human sepsis.

Other researchers in the lab have found a similar fall in cardiac output. However, they found clear differentiation between eventual survivors and non-survivors in terms of stroke volume (lower in non-survivors) and heart rate (higher in non-survivors) which I did not find. Whereas stroke volume was non-significantly lower in eventual non-survivors, heart rate was unchanged and trended lower than either septic survivors or healthy controls. This may relate to my echocardiographic measurement technique, however in my training phase I recorded images from which I made the stroke volume and heart rate measurements and these were validated by experienced members of the lab. Another possibility is that I may have been more liberal in the use of anaesthesia to allow enable echocardiographic measurement and this may have had a larger depressant effect on heart rate in the sicker animals. An alternative explanation is that the phenotype of the animals or the lab climate changed. For example, core temperature was previously noted in this model to be maintained or elevated at 6h despite falls in oxygen consumption compared to healthy controls, whereas temperature dropped in my septic animals, more so in septic non-survivors.

Clinical scoring was a good indicator of survival, especially the animal's breathing pattern. Although a late marker, animals with laboured breathing were likely to be non-survivors as most failed to improve and had to be culled to not exceed the severity limit.

In terms of metabolism, the normal diurnal rhythm in RER (ratio of CO_2 production to O_2 consumption) seen in healthy animals was lost in the septic animals and did not recover by 72 hours in the survivors, despite clinical recovery. This has been repeatedly seen in this 72-hour lab model and even in a longer-term 7 day model of zymosan peritonitis [419]. In the septic animals, there was a reduction in both CO_2 production and O_2 consumption compared to healthy controls which was greater (albeit non-significantly) in the non-survivors. While this will reflect in part the deceased motor activity in these animals who were feeling unwell following the

septic insult, it is notable that these values did not recover despite clinical improvement in survivors by 72 hours where the animals had clinically improved, were more mobile and eating/drinking better.

Studies dating from the 1980s and 1990s investigated systemic oxygen delivery and oxygen consumption in sepsis and trauma and reported lower levels in eventual non-survivors [420-422]. While early studies suggested improved survival using fluid and inotropes to augment oxygen delivery [420-422], this was not borne out by prospective studies including large multinational prospective trials [423].

After induction of sepsis, RER values fell from a baseline value of 0.9 to 0.8 after 5 hours and stayed around this level for the remainder of the study in both survivor and non-survivor animals. On the other hand, RER values in the sham animals ranged between 0.9-1.0 with a much greater diurnal swing reflective of their eating habits and greater nocturnal activity. An RER of 1.0 reflects pure carbohydrate metabolism while 0.7 reflects pure fat metabolism. My data confirm a shift towards fat metabolism which is well described in critical illness, as discussed in the Background chapter.

5.5.2 Safety study

A metabolic alkalosis was seen with butyrate in both the anaesthetised animals and the awake, longer-term model. The butyrate solution infused had a pH of 7.7, whereas the control glucose/Hartmann's solution was acidic. Furthermore, unlike the control solution, sodium butyrate has buffering capacity, together explaining the alkalotic effect. Fluids generally given to patients have a lower pH, e.g. 5.0-7.0 for Hartmann's solution, 4.0 for 5% glucose and 5.5 for n-saline. Due to time pressures, I did not study the role of the pH difference on clinical, echocardiological and metabolic outcome. I hence did not adjust the pH of either the butyrate solution or control solution to equalise.

Animals did however tolerate this dose of butyrate, and the RER data showed a clinical impact of the butyrate infusion by lowering RER. I therefore decided to continue using this dose for the butyrate infusion studies as the animals did not

appear to be compromised clinically and I wanted to achieve a plasma concentration equivalent to what was used *in vivo*. Furthermore, sepsis often induces a metabolic acidosis so this would be counteracted to some degree by the alkaline solution.

5.5.3 Butyrate infusion study

A butyrate infusion was given to impact on immune and mitochondrial function *in vivo*. The rationale for using butyrate was based upon my findings described in Chapters 3 and 4 showing anti-inflammatory and mitochondrial stimulatory effects of butyrate on PBMCs. These effects were also supported by other reported *in vitro* and *in vivo* studies, as discussed in these chapters. For example, SCFA and butyrate supplementation were shown to increase survival in septic rats [424] and mice [425], and to reduce inflammation in both septic rats [425] and mice [426].

Many of these studies however differed in setup, or did not combine immune with mitochondrial function. Butyrate has been given by injections rather than infusion [424], or as pretreatment, either in the feeding regimen [426] or intraperitoneally [427]. In a clinical scenario treatment would not commence until after the patient presents with sepsis. Some studies have used mice [426, 427] or have studied the effects of butyrate but not in combination with sepsis [428]. Infusion studies in septic rats did not use SCFAs but different fatty acids [429, 430].

Some human studies have also been performed, but only a few have examined SCFA supplementation in sepsis, with the focus on microbiome dysbiosis [431]. A human trial on the effects of butyrate on inflammatory potential of PBMCs gave oral supplementation of butyrate, and did not focus on sepsis [432]. Many studies and clinical trials have been performed on the metabolic and immune impacts of intravenous fat emulsions in critical illness, but these infused emulsions mostly contain MCFAs and LCFAs and not SCFAs [170, 433].

5.5.3.1 *Metabolic monitoring*

VO_2 and VCO_2 levels (Figure 5-7 and Figure 5-8 respectively) of naïve animals were significantly higher compared to septic animals. Although no difference in VO_2

consumption was measured after onset of butyrate and control solution infusion, the butyrate infused animals had a bigger decrease in VCO_2 levels compared to the control group. As a consequence, the RER of septic animals was significantly lower in septic compared to naïve animals, and lower still in those receiving butyrate (Figure 5-9). This suggests that butyrate infusion indeed induces a shift towards fat metabolism, and that butyrate could be used as a caloric source.

A study on mice on a high-fat diet supplemented with sodium butyrate also showed a reduction in RER on butyrate supplementation, as well as a modest increase in VO_2 which was not shown in my study. This study was however in the context of obesity rather than sepsis, and metabolic cart data were obtained at week 1 and week 10 of butyrate supplementation at 5% wt/wt [405].

Between two and six hours after faecal slurry injection, animals received 1:1 glucose/Hartmann's solution. Treatment started after six hours, with sodium butyrate and control solutions isocaloric and isovolumic. This was achieved by adding 1.5x more 5% glucose solution to the control solution, to compensate for the extra calories (0.49 kcal/kg/h) given by adding sodium butyrate to the 1:1 glucose/Hartmann's solution.

According to ESPEN guidelines [434], ICU patients should receive supplemental parenteral nutrition to fully cover their needs and decrease negative energy balance when enteral feeding is contraindicated, inadequate or failing. ICU patients are recommended to receive 25 kcal/kg/day. The exact amount of calories a patient needs is however difficult to define and varies over time. Important parameters involve the nutrition status of the patient, days of hospitalisation, endogenous nutrient production and autophagy, energy balance and the occurrence of refeeding syndrome [435]. ESPEN states that lipids should be an integral part of parenteral nutrition, but clinicians should mostly consider intravenous lipid emulsions containing long-chain triglycerides (LCT), medium-chain triglycerides (MCT) or mixed emulsions and not SCFAs like I used here. Lipid emulsions of LCT and MCT can safely be administered at a rate of 0.7 - 1.5 g/kg over 12-24 hours [434].

ESPEN also recommends a minimum 2 g/kg/day of glucose, but hyperglycaemia (glucose >10 mmol/l) should be prevented To compare, animals used in my studies received 0.25 g/kg/h glucose as a 1:1 glucose/Hartmann's solution, i.e. 6 g/kg/day. Septic control animals received 0.38 g/kg/h (=9 g/kg/day) to cover the extra calories given by adding sodium butyrate to the 1:1 glucose/Hartmann's solution, thus animals received about 42 kcal/kg/day. Basal metabolic rate in rats is however about 6.4 times higher than in humans [436]. Furthermore, septic rats used here, unlike most septic patients, are young and without comorbidities, making their energy requirements different.

The rats received a high carbohydrate load in the form of glucose. The normal response to carbohydrate load in excess of energy expenditure is a rise in RER to 1.0 or greater [437], indicating glucose is the main substrate used oxidised for energy. As shown in Figure 5-9, septic rats had a lower RER even in presence of high glucose load. In a study dating from 1980 [437], hypermetabolic injury and septic patients received large glucose load. Rather than suppressing fat oxidation, fat oxidation was continued and an increase in the conversion of glucose to glycogen was found. Injured/infected patients further had an increase CO_2 production, which may compromise the patient with borderline pulmonary reserve. Furthermore, these hypermetabolic patients had increased urinary catecholamine excretion and increased energy expenditure. The large carbohydrate intake may serve as a physiological stress rather than nutritional support [437].

As discussed in section 1.7, sepsis is characterised by early massive catabolism [438, 439], lean body mass loss [440] and escalating hypermetabolism persisting for months to years [441]. The acute metabolic response to stress can generate 50-70% of glucose needs during illness [439] and is not suppressed by feeding or intravenous glucose infusion [442]. Caloric need does not consistently increase in the early acute phase of sepsis, as shown by a total energy expenditure to resting energy expenditure ratio of 1.0 in sepsis [443]. Moreover, resting energy expenditure is lower in more severe septic shock, as the body hibernates and reduces metabolism [126]. An increasing body of data suggests we should consider feeding less nonprotein calories early in the acute phase of sepsis of critical illness

and considerably increase calorie delivery during recovery [443-445]. Therefore, the current model has likely administered too much glucose in both septic control and septic butyrate infused animals and this should be improved in future studies.

On the other hand, there is also increasing evidence that persisting underfeeding throughout ICU stay, in particular protein underfeeding, considerably contributes to long-term mortality and impairment of life quality months later [442, 446-448]. Early enteral nutrition should therefore attempt to deliver adequate protein. Furthermore, enteral nutrition should attempt to correct micronutrient/vitamin deficiencies. Both of these aspects have not been addressed in the present model. Nutritional therapy after resuscitation should further increase protein and calorie delivery to attenuate lean body mass loss and promote early mobility and recovery, which should continue after ICU discharge [441].

5.5.3.2 Echocardiography and blood gases

Blood gas data indicates signs of metabolic alkalosis as shown by a rise in pH, base excess and bicarbonate, and a compensatory respiratory acidosis. This is similar to what was observed in the safety study. The increase in sodium is likely to be related to the infusion of the drug, namely sodium butyrate. I gave 540 mmol/l sodium as sodium butyrate, in addition to the 65.5 mmol/l sodium present in the 1:1 glucose/Hartmann's solution. The septic control animals did not receive the 540 mmol/l of extra sodium.

The rise in pH may explain the rise in lactate. Acid-base disturbances (changes in pH) have various metabolic consequences, including affecting Krebs' cycle, oxidative reactions, gluconeogenesis and the hexose monophosphate shunt [449]. MacLeod and Hoover were the first to observe in 1916 [450] that alkalosis, either metabolic or respiratory, increases aerobic production of lactate. The rise in lactate production as result of increased pH is primarily, but not exclusively, linked to the sensitivity of phosphofructokinase, an enzyme involved in the sequential conversion of glucose to lactate (Embden-Meyerhof pathway) and enhancing glycolysis. Both anoxia and pH are major factors in controlling glycolysis [449].

The concentration of pyruvate in the cytoplasm is in equilibrium with lactate via the lactic dehydrogenase reaction. This pyruvate concentration is mainly determined by the rate of its formation through the Embden-Meyerhof pathway, the rate by which pyruvate is utilised in the Krebs cycle and by the rate at which it is utilised in the pyruvic carboxylase and phosphor-enol-pyruvate carboxykinase pathways that leads to gluconeogenesis. Effects of alkalosis in blocking mitochondrial oxidation and Krebs' cycle turnover have been suggested [451]. Also Krebs' cycle intermediate are affected by pH, and it is generally recognised that concentration of citrate in various tissues is reduced in acidosis and increased in alkalosis [452-455].

Alternatively, or in part, the rise in lactate may reflect worse tissue perfusion and/or mitochondrial impairment. Cardiac output in my study was significantly lower in butyrate-treated septic animals compared to septic controls so this is a distinct possibility. Depressed cardiac function is a major complication in sepsis [100]. Even though cardiac output may be elevated, there is decreased responsiveness to inotropic agents such as dobutamine [456]. In my study, where butyrate was commenced after 6 hours of sepsis, stroke volume was not improved over non-treated septic controls and cardiac output was lower. In a study on LPS-challenged mice receiving a butyrate diet, butyrate attenuated septic myocardial depression via an anti-inflammatory pathway and reduction in oxidative stress [425]. The authors however used butyrate as a pretreatment (200mg/kg/day i.p. for 3 days) prior to an i.p. LPS challenge. Their dose was also lower than that used in my study (0.2 g/kg/day compared to 0.6 g/kg/h). Heart function was assessed by left ventricular function *in vivo* and myocardial tissue damage by oxidative stress markers. Although I could not show an improvement in heart function based on stroke volume, cardiac output and heart rate, heart tissue and plasma samples were stored and could be assessed for other myocardial functionality and markers oxidative stress such as isoprostanes and protein carbonyls.

Glucose levels in the septic controls were lower than healthy controls and this was restored with butyrate. This may relate to a shift in utilisation towards fat and decreased utilisation of glucose. This was also reflected by a higher RER of septic control animals compared to butyrate-infused animals. The RER dropped further in

the butyrate-treated group, indicative of fat metabolism, less use of glucose and hence higher glucose blood levels. Furthermore, septic control animals received no fat in their parenteral nutrition, but only glucose. Although animals had free access to their standard chow food, septic animals tend not to eat within 24 hours after sepsis onset. Septic control animals would therefore rely more on glucose plus endogenous fat and protein stores for metabolism, whereas butyrate-infused animals could also use the additional fatty acids. Of note, glucose levels were similar to naïve animals and hence no glucose toxicity was seen.

Hepatic glucose production is autoregulated during infusion of gluconeogenic precursors, but this may be defective in hyperglycemic patients with multiple trauma. In a study, healthy volunteers were infused with glucose alone and glucose plus lactate. Glucose alone nearly abolished endogenous glucose production, whereas lactate increased gluconeogenesis. In patients with metabolic stress however, exogenous lactate did not suppress endogenous glucose production, but lactate did not further increase hepatic glucose production [457]. These findings are in line with my findings shown in Figure 5-10, where I find increased lactate together with increased glucose in the septic butyrate infused group, suggesting a defective autoregulation of hepatic glucose production in the presence of glucose administration.

In another study, five male patients with multiple trauma were infused with a solution with 65% of total calories derived from carbohydrate, 15% lipid and 20% amino acids (high glucose). Afterwards, they received solution with calories derived for 35% from carbohydrate, 30% lactate, 20% lipid, 15% amino acid (high lactate). Compared to the high glucose solution, the lactate solution decreased glycemia by 20%, net carbohydrate oxidation by 34% and plasma oxidation by 54%. Arterial pH increased from 7.41 to 7.46. This data suggests a glucose oxidation sparing effect of lactate. Together, the authors concluded that sodium lactate as a metabolic substrate limits hyperglycemia, but induces metabolic alkalosis [458].

The effect of butyrate infusion in sepsis in the form of ketone body betahydroxybutyrate was assessed in a study in dogs. As opposed to nonseptic

dogs, infusion of betahydroxybutyrate in septic dogs did not suppress either glucose production or plasma FFA, suggesting a loss of normal regulatory actions of ketones [459]. This data again shows a glucose sparing effect of butyrate, which I saw in my study too.

The higher glucose levels in the butyrate treated group may also possibly explain in part the rise in lactate. Decreased mitochondrial utilisation of pyruvate, the end-product of glycolysis, with a corresponding rise in intracellular levels, will increase lactate levels as pyruvate and lactate are in equilibrium.

In my study, glucose levels were lower in septic control animals, but restored to naïve animal glucose levels with butyrate infusion. Multiple studies show an association between sodium butyrate and improved insulin sensitivity, as reviewed in [460]. If so, glucose levels should have fallen if insulin sensitivity was improved by sodium butyrate administration in the septic animals but this was not seen. However, most of these studies do not assess butyrate infusion, but administer butyrate via a dietary intake and also for a longer duration (days to weeks). Furthermore, the impact on insulin sensitivity is tissue dependent [460], and these studies focussed on SCFAs in colon cancer and insulin resistance in obesity and not sepsis. In a rat model of parenteral nutrition, a parenteral mixture of SCFAs increased ileal glucose transporter 2 (GLUT2) and proglucagon mRNA expression more than butyrate fed rats alone. The authors suggested that the effects of SCFAs on intestinal gene expression may not be butyrate specific [461]. Additionally, a study on beta-hydroxybutyrate infusion in dogs concluded effects on glucose and FFA metabolism were independent of changes in insulin and glucagon levels and sympathetic activity. Regardless of hormonal status, authors showed that elevated ketone levels following beta-hydroxybutyrate infusion in dogs decreased the rate of glucose production and plasma FFA levels [462].

5.5.3.3 Mitochondrial respiration of splenocytes

Basal respiration in splenocytes isolated from the septic animals was reduced in those given butyrate. This appears to be due to an overall depressant effect on cellular metabolism as there was a reduction in ATP-linked mitochondrial

respiration and proton leak, and in non-mitochondrial respiration. By contrast, proton leak increased in the septic animals not treated with butyrate. The reduction in maximal respiration was however similar in both septic groups, suggesting a change in membrane potential rather than decreased proton conductance as an underlying mechanism for reduced mitochondrial respiration in butyrate-treated septic animals. Splenocyte viability was over 90% as confirmed by live/dead stain using flow cytometry. Overall, these results are different from the effects of butyrate in my *in vitro* study demonstrated in Chapter 4, where I showed an increase in maximal respiration and spare respiratory capacity of PBMCs exposed to butyrate.

Reasons for this disparity are unclear. As mentioned in the introduction of this discussion (section 5.5), similar studies to mine are scarce. Studies with a different setup, given to different disease processes and assessing different aspects of mitochondrial function are published but do not permit direct comparison. Furthermore, respirometry data on rat splenocytes are scarce; I only found one study with a similar setup albeit looking at hemorrhagic shock [463].

A study on butyrate-supplemented rats showed an increased expression of PGC-1α, an enzyme important in mitochondrial biogenesis and function. The authors however studied hepatocytes and compared this against a high-fat diet to simulate obesity [464]. Another study compared the transcriptome and proteome of colonocytes of germ-free mice with normal mice, and identified an important role for microbiota and its metabolites (butyrate in particular) in mitochondrial function and metabolism in the colon [404]. Based on transcriptomic and proteomic data, germ-free mice had decreased fatty acid oxidation, as shown by decreased levels acyl CoA dehydrogenase and a 31% decrease in ^{13}C-butyrate conversion to labelled CO_2 compared to normal mice. Furthermore, downregulation of pyruvate dehydrogenase and decreased ATP levels were shown. TCA cycle enzymes were also downregulated, which resulted in diminished a $NADH/NAD^+$ ratio in the mitochondria. The authors further demonstrated an important role for butyrate in mitochondrial respiration of colonocytes, as colonocytes from germ-free mice exhibited a marked decrease in mitochondrial respiration, but no difference in total

mitochondria. These effects were attributed to butyrate as colonisation of germ-free mice with a butyrate-producing strain rescued oxidative phosphorylation, ATP levels and autophagy. Butyrate dramatically increased oxidative phosphorylation in germ-free colonocytes (from 30% to 70%) [404].

A study on mice found that supplementation of butyrate to a high-fat diet increased fatty acid oxidation, mitochondrial function and biogenesis in skeletal muscle and brown fat [405]. There was also increased PGC-1α expression at mRNA and protein levels, increased UCP-1 in brown adipose tissue, and a rise in AMP kinase, p38, mitochondrial DNA, and CPT-1.

Most studies find increased mitochondrial function upon butyrate exposure, but I found a decrease in mitochondrial function in the splenocytes taken from butyrate-treated animals. A study on cell lines from autistic boys showed that butyrate dose-dependently decreased mitochondrial respiration as shown by a decrease in ATP-linked, proton leak maximal and spare respiratory capacity, with the highest dose (1 mM) having most impact [465]. The authors however also studied a lower dose of 0.1 mM butyrate which non-significantly increased these respiratory parameters. Opposite results were shown when oxidative stress was induced: butyrate dose-dependently increased mitochondrial function. My data show an increase in mitochondrial ROS production upon butyrate infusion (Figure 5-13), but no improvement in mitochondrial function. The levels of ROS may have been too high, inducing harm, as discussed later.

The authors further reported that the oxygen consumption rate of different lymphoblastoid cell lines had marked differences [465], indicating that responses are cell type and even cell line specific, making it increasingly difficult to compare different studies.

The dose-dependent effect described above suggests that the butyrate dose I infused could have been too high. Future studies should therefore include different doses, and plasma butyrate concentrations should ideally be determined. Furthermore, as mitochondrial function is tightly regulated by pH [466], the pH difference in control (pH 5.5 – 6.0) versus butyrate (pH 7.7) should be assessed. The

marked increase in arterial base excess and corresponding increase in $PaCO_2$ may have impacted on cellular function. Viability of splenocytes was however not affected as measured by live/dead stain using flow cytometry. Due to lack of time, I have not been able to study these aspects. Given the *in vitro* stimulatory effects of butyrate on mitochondrial function I showed in PBMCs, further optimisation of this *in vivo* model would be meaningful.

5.5.3.4 Flow cytometry studies on splenocytes

Butyrate increased membrane potential and induced a 10-fold increase in mitochondrial ROS production over untreated septic controls, indicative of increased mitochondrial stress and damage resulting in a fall in membrane potential. Fatty acids, especially LCFAs, reduce ROS generation due to their weak inhibition of electron flow at complexes I and III [247] and their protonophore action on the inner mitochondrial membrane [467]. The protonophore effect however decreases as chain length decreases. Butyrate, with a chain length of 4 carbon atoms, may therefore not have much protonophore activity. However, butyrate significantly lowered ATP turnover while significantly increasing mitochondrial oxygen consumption in perfused liver isolated from fed rats [468], suggestive of increased uncoupling. Uncoupling effects of SCFAs on immune cells have also been shown [407], however low physiological levels of butyrate did not affect functioning of the electron transport chain nor did it have any protonophore effects on the inner mitochondrial membrane [247]. On the other hand, excessive accumulation of MCFAs seen with inborn medium-chain acyl-CoA dehydrogenase deficiency [469] is associated with inhibition of mitochondrial respiratory chain complexes [470], possibly leading to increased ROS production. Although I did not measure plasma butyrate levels, I used a dose reported in pharmacokinetic studies to generate a concentration of 1.8 mM [414]. The negative effects, including increased mitochondrial ROS production, suggests this dose was excessive and therefore harmful.

In terms of cytokine production by the splenocytes *ex vivo*, butyrate had no impact on the raised TNF levels seen in the septic cells, but there was a trend towards an

increase in IL-10 production. These results differed from my *in vitro* studies where I noted reduced secretion of IL-10 by PBMCs exposed to butyrate (Figure 3-2). Although butyrate treatment did not lower TNF, it also did not increase inflammation given the marked increase in ROS production described above. TNF and ROS influence each other in a positive feedback loop [471]. ROS at low concentrations regulates TNF signalling, but concentrations here were probably too high and may have caused toxicity.

A study on the immunomodulating effects of butyrate on murine splenocytes showed that butyrate inhibited CD4+ and CD8+ T cell activation upon anti-CD3 stimulation, however only at 5 mM concentration and not at 3 mM [472]. The study also showed an inhibitory effect of butyrate on T cell proliferation, and enhanced apoptosis. As described in Chapter 3, different effects of butyrate on IL-10 secretion have been reported. Some show an increase in IL-10 production [383]. The stimulatory effects of butyrate on regulatory T cells [390] could explain the increase in IL-10 production shown in splenocytes in my studies. For future experiments, after dosing has been optimised, it may be worth adding specific T cell markers to discriminate between different types of T cells. Monocytic markers may be of interest too as they too are members of the innate immune system and producers of TNF. Unfortunately, rat antibodies for flow cytometry are still very limited.

Of note, cytokine analyses by flow cytometry measures intracellular cytokine production and not cytokine secretion. I could not detect notable cytokine levels in plasma, as discussed below. Furthermore, I analysed splenocytes rather than circulating PBMCs. Future studies could focus on other immune cell types for flow cytometric analyses, for example PBMCs (although lower in count) or whole blood.

5.5.3.5 *LPS and PMA stimulation*

No or low cytokine levels were measured both in plasma from the septic animals and in splenocyte supernatant after overnight stimulation with LPS and PMA. Though unlikely, splenocytes could have lost their functionality. Low/unrecordable plasma cytokine levels have been recently seen by other researchers in the lab using the same septic animal model; this is a departure from previous findings and,

as yet, unexplained. There are ongoing efforts to try to explain this discrepancy as the model is otherwise unchanged in terms of clinical phenotype and illness severity.

5.6 Conclusion

Based on *in vitro* studies using PBMCs, I expected butyrate to have a positive impact on sepsis in reducing cytokine secretion and increasing ATP-linked and maximal respiration. This could not however be demonstrated in this *in vivo* model of sepsis. Blood gas, echocardiography and mitochondrial parameters indicate increased stress upon butyrate infusion, and butyrate did not significantly modulate *ex vivo* cytokine production in septic splenocytes. The septic animals clinically looked the same, but the above-mentioned variables clearly showed that they were more compromised. A reduction in RER and hence switch towards fat metabolism indicates that butyrate was being used as a substrate.

The current study was well characterised and the methodology was appropriate, using various techniques, good controls (albeit not a butyrate-treated non-septic control) and measuring multiple parameters. The negative effects of butyrate may be related in part to its alkalotic effects, or to direct toxicity. I was unable to measure plasma butyrate levels as this requires liquid gas chromatography which was not available and so I relied on a previously reported pharmacokinetic study [414] to deliver a dose that delivered the desired plasma concentration. This may have been excessive. As these experiments were done at the end of my research time, I was not able to do dose-finding studies to see if lower doses would have been beneficial, or whether neutralising the alkalosis (either in the solution administered or *in vivo*) would have made a difference. Similarly, I would have liked to perform flow cytometry on PBMCs to compare mitochondrial markers and intracellular cytokine production by circulating immune cells against those measured in splenocytes. Additionally, I would add more cell specific markers to the splenocyte flow cytometry experiments, for example markers for regulatory T cells or monocytes. However, unlike human and mice flow cytometry antibodies, those for rats are very limited.

Chapter 6   Conclusions and future work

This study examined the impact of fatty acids in modulating metabolic, immune and mitochondrial function in sepsis using *in vitro* studies on stimulated PBMCs and an *in vivo* rat model of faecal peritonitis. Methodology was carefully considered prior to all experiments, and *in vitro* studies were performed at first to get insight in the effects of palmitate, butyrate and alpha-linolenic acid on immune and mitochondrial function without sacrificing animals.

In vitro experiments using human PBMCs showed that palmitate, butyrate and alpha-linolenic acid have proinflammatory, anti-inflammatory and mixed effects on immune function. Effects on palmitate and butyrate are largely in accordance with literature, although alpha-linolenic acid is mostly known to be anti-inflammatory. These effects indicate that palmitate and butyrate may both have potential in treating sepsis, but in different phases. Butyrate might be effective in the early pro-inflammatory phase of sepsis, whereas palmitate may stimulate immune responses in the later immunosuppressed state.

In terms of mitochondrial respiration, butyrate seemed most promising in sepsis by increasing both maximal respiration and spare respiratory capacity. Butyrate thus makes PBMCs more able to respond to stress caused by sepsis. Palmitate increased basal respiration and proton leak, but unlike other studies not maximal respiration. Alpha-linolenic acid unexpectedly decreased mitochondrial respiration and might cause toxicity to the cells. Given these results, I decided butyrate at a dose of 1.8 mM to take forward in an *in vivo* model of sepsis.

The animal model was carefully characterised to ascertain that the rats would reach the desired study endpoint and could be treated. A faecal slurry dose-finding study as well as a butyrate infusion dose-finding study was performed with careful monitoring of clinical severity, cardiac and metabolic function to optimise methodology.

Butyrate infusion at a rate of 0.6 g/kg/h resulted in a decreased RER, indicating a shift towards fat metabolism. However, effects of butyrate shown in *in vitro* studies

on immune and mitochondrial function could not be reproduced in this animal model of sepsis. Compared to naïve control animals, mitochondrial respiration was decreased in splenocytes isolated from septic rats, and even further in those treated with butyrate. Butyrate treatment also increased mitochondrial ROS production by a 10-fold, indicative of oxidative stress. Although sepsis increased intracellular TNF production in splenocytes, butyrate infusion had no effect. Butyrate showed anti-inflammatory properties by increasing intracellular IL-10. Unfortunately, no cytokine secretion could be detected after overnight stimulation with LPS and PMA. Butyrate infusion did not increase cardiac function in the present study and did not impact upon clinical severity scoring.

Blood gas data showed a metabolic alkalosis in the treated group. The treated animals received a sodium butyrate solution with a higher pH than the control solution given to the non-butyrate-treated animals. For future experiments I would tackle the pH issue, as well optimising nutritional strategy. Dosing should be optimised too, with use of liquid gas-chromatography to determine plasma butyrate concentrations. After further optimisation of the present of the model, I would also increase the group size for larger studies.

I did not investigate mechanisms in this thesis. Post-mortem tissues were however sampled and these could be studied for UCP and GPR expression for mechanistic insights. These mechanisms, as well as NF-κB and/or HDAC expression, could be assessed in PBMC analyses too. Future studies could also examine flow cytometric analyses of circulating immune cells in whole blood, which would be quicker and more clinically relevant than isolated splenocytes. However, the number of cells obtained would not be sufficient for concurrent respirometry analyses which is why I chose to use splenocytes. Future studies might also include study of other fatty acids, alone or in combination.

The current study provided cell-specific flow cytometry panels on PBMCs with working methodology. This could form the basis of future work in sepsis research on PBMCs, although adding mitochondrial markers to these panels may have yielded interesting data. In retrospect, I would have reduced the number of

conditions to make room for mitochondrial markers and more replicates on T cell functionality.

The *in vitro* studies on PBMCs looked promising and especially effects on butyrate were largely in line with existing literature. Yet, I demonstrated that *in vitro* studies cannot easily be translated to what happens *in vivo*. The effects of butyrate shown *in vitro* and *in vivo* however reconfirmed a role for nutrition besides providing a caloric source. I here demonstrated that fatty acids also impacts on immune and mitochondrial function.

Additionally, this thesis underscored that a one-size-fits-all approach does not always apply. By characterising the patient's specific needs by using a combination of parameters, treatment could be allocated for each individual to benefit the patient, also known as 'personalised medicine'. The septic animals clinically looked the same, though the immune and metabolic parameters showed they were not. This emphasises a need for personalised medicine.

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