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Akademisk avhandling

# Mitochondrial function in sepsis

## Temporal evolvment of respiratory capacity in human blood cells

av

**Fredrik Sjövall**

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i Belfragesalen, Biomedicinskt Centrum

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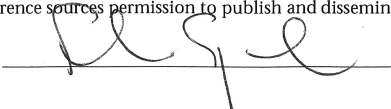
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<p><b>Abstract</b></p> <p>Sepsis is a devastating disease that is caused by the host's response to an overwhelming infectious process. As sepsis progresses, organs distant from the site of infection become affected and sepsis-induced multiple organ failure ensues. An impaired immunologic response, including dysfunctional peripheral blood immune cells has been described as part of the septic syndrome. Mitochondrial dysfunction has been suggested to be a contributing factor in the pathogenesis of these alterations and restoration of mitochondrial function has been implicated as a prerequisite for the recovery from sepsis. Further, platelets have been proposed to serve as a surrogate tissue in evaluation of systemic mitochondrial dysfunction. The overall aim of this thesis was to evaluate the temporal evolution of mitochondrial respiratory function in platelets and peripheral immune cells during the course of sepsis.</p> <p>In the first study we established methodology and performed a thorough assessment of normal human platelet respiratory function ex vivo from healthy individuals in a wide age-span using high-resolution respirometry. We concluded that freshly isolated platelets, intact or permeabilised, were well suited for studying human mitochondria ex vivo. With different titration protocols, detailed information of the cellular respiratory capacities could be obtained and we deemed this approach suitable for evaluating endogenous mitochondrial capacity as well as alterations of mitochondrial function induced by exogenous factors.</p> <p>In the two subsequent studies we examined mitochondrial respiratory function in platelets and peripheral blood immune cells (PBICs) of patients with severe sepsis or septic shock and studied its evolvement during the first week following admission to the intensive care unit. In both cell types we found that mitochondrial respiration (per cell) gradually increased during the week analysed. In platelets, this increase was higher in patients who subsequently died. Also, in platelets, we observed reduced respiratory control ratios of intact platelets when the cells were suspended in the patient's own plasma. As markers for mitochondrial content we measured mitochondrial DNA (mtDNA), cytochrome c (Cyt c) and citrate synthase (CS). There was a difference between the two cell types in that the markers were profoundly more increased in PBICs compared to platelets even though they displayed approximately the same levels of increase in mitochondrial respiration.</p> <p>In the final study of this thesis we evaluated cytokines and nitric oxide in the plasma from the septic patient cohort since these signaling molecules have been demonstrated to enhance mitochondrial respiration through stimulation of mitochondrial biogenesis. Of ten different cytokines and NO analysed, IL-8 levels correlated positively with both maximal ATP-generating as well as maximal non-ATP-generating rates of respiration in samples from the latest time point evaluated. Further, the plasma level of IL-8 was higher in non-survivors in samples taken at day 6-7 compared to survivors.</p> <p>In conclusion, this thesis demonstrates that circulating blood cells exhibit increased respiratory capacities throughout the first week of sepsis. This increase seems to be accomplished by different mechanisms; in PBICs by increased mitochondrial mass as indicated by elevated levels of mitochondrial markers, and in platelets possibly by a post-translational regulation of mitochondrial respiratory capacity. In addition, a plasma factor seems to be able to induce increased uncoupling of respiration in platelets during sepsis.</p>		
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# Mitochondrial function in sepsis

Temporal evolution of respiratory capacity in human  
blood cells

Doctoral Dissertation

**Fredrik Sjövall**

2013

Mitochondrial Pathophysiology Unit  
Department of Clinical Sciences  
Faculty of Medicine



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Poul Anderson



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# ORIGINAL PAPERS

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

- I. Sjövall F., Ehinger J.K., Marelsson S.E., Morota S., Åsander-Frostner E., Uchino H., Lundgren J., Arnbjörnsson E., Hansson M.J., Fellman V., Elmér E. Mitochondrial respiration in human viable platelets - Methodology and influence of gender, age and storage.  
*Mitochondrion 2013; 13:7-14.*
- II. Sjövall F., Morota S., Hansson M.J., Friberg H. Gnaiger E., Elmér E. Temporal increase of platelet mitochondrial respiration is negatively associated with clinical outcome in patients with sepsis.  
*Critical Care 2010; 14:R214.*
- III. Sjövall F., Morota S., Hansson M.J., Elmér E. Patients with sepsis exhibit increased mitochondrial respiratory capacity in peripheral blood immune cells.  
*Submitted for publication.*
- IV. Sjövall F., Morota S., Åsander-Frostner E., Hansson M.J., Elmér E. Cytokine and nitric oxide levels in patients with sepsis and their correlation with mitochondrial respiratory function.  
*Submitted for publication.*

## **Related publication**

Sjövall F., Hansson M.J., Elmér E. Platelet mitochondrial function in sepsis.  
*Crit Care Med 2012; 40:357; author reply 357-358.*

# ABBREVIATIONS

ACD - acid citrate dextrose	NET - neutrophil extracellular traps
ANT - adenine nucleotide translocator	NF- $\kappa$ B - nuclear factor- $\kappa$ B
AT-III - anti-thrombin III	NLRs - nucleotide-binding domain, leucine rich repeat containing proteins
CARS - compensatory anti-inflammatory response syndrome	NO - nitric oxide
CI - complex I	NOS - nitric oxide synthase
CII - complex II	NRF - nuclear respiratory factor
CIII - complex III	O <sub>2</sub> <sup>-</sup> - superoxide
CIV - complex IV	OH <sup>•</sup> - hydroxyl radical
CoQ - ubiquinone	ONOO <sup>-</sup> - peroxynitrite
CS - citrate synthase	OXPHOS - oxidative phosphorylation
Cyt c - Cytochrome c	PAMPs - pathogen-associated molecular patterns
DAMPs - danger associated molecular patterns	PARP - poly-(ADP-ribose) polymerase
DIC - disseminated intravascular coagulation	PBICs - peripheral blood immune cells
ETS - electron transport system	PBS - phosphate buffered saline
FAD - flavin adenine dinucleotide	PDH - pyruvate dehydrogenase
FCCP - carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone	PGC-1 $\alpha$ - peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) co-activator 1- $\alpha$
FFA - free fatty acids	PRP - platelet rich plasma
H <sub>2</sub> O <sub>2</sub> - hydrogen peroxide	PRR - pattern recognition receptor
HMGB-1 - high-mobility group box-1 protein	RAGE - receptor for advanced glycation end-products
ICU - intensive care unit	RLR - retinoid acid-inducible gene I receptor
IL - interleukin	RNS - reactive nitrogen species
INF $\gamma$ - interferon- $\gamma$	ROS - reactive oxygen species
IQR - interquartile range	SIRS - systemic inflammatory response syndrome
LPS - lipopolysaccharides	SUIT - substrate, uncoupler, inhibitor titration
MACF - mitochondrial anion carrier family	TCA - tricarboxylic acid
MCP-1 - monocyte chemotactic protein-1	TF - tissue factor
MFA - mitochondrial fractional area	TFAM - mitochondrial transcription factor
MIF - macrophage migration inhibitory factor	TFPI - tissue factor pathway inhibitor
MiR05 - mitochondrial respiration medium	TLR - Toll-like receptors
MnSOD - manganese superoxide dismutase	TMPD - N,N,N',N'-tertamethyl-p-phenyldiamine
MODS - multiple organ dysfunction syndrome	TNF - tumor necrosis factor
mPT - mitochondrial permeability transition	UCP - uncoupling proteins
mtDNA - mitochondrial DNA	
NAD - nicotinamide adenine dinucleotide	
nDNA - nuclear DNA	

# SUMMARY

Sepsis is a devastating disease that is caused by the host's response to an overwhelming infectious process. As sepsis progresses, organs distant from the site of infection become affected and sepsis-induced multiple organ failure ensues. An impaired immunologic response, including dysfunctional peripheral blood immune cells has been described as part of the septic syndrome. Mitochondrial dysfunction has been suggested to be a contributing factor in the pathogenesis of these alterations and restoration of mitochondrial function has been implicated as a prerequisite for the recovery from sepsis. Further, platelets have been proposed to serve as a surrogate tissue in evaluation of systemic mitochondrial dysfunction. The overall aim of this thesis was to evaluate the temporal evolution of mitochondrial respiratory function in platelets and peripheral immune cells during the course of sepsis.

In the first study we established methodology and performed a thorough assessment of normal human platelet respiratory function *ex vivo* from healthy individuals in a wide age-span using high-resolution respirometry. We concluded that freshly isolated platelets, intact or permeabilised, were well suited for studying human mitochondria *ex vivo*. With different titration protocols, detailed information of the cellular respiratory capacities could be obtained and we deemed this approach suitable for evaluating endogenous mitochondrial capacity as well as alterations of mitochondrial function induced by exogenous factors.

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generating rates of respiration in samples from the latest time point evaluated. Further, the plasma level of IL-8 was higher in non-survivors in samples taken at day 6-7 compared to survivors.

In conclusion, this thesis demonstrates that circulating blood cells exhibit increased respiratory capacities throughout the first week of sepsis. This increase seems to be accomplished by different mechanisms; in PBICs by increased mitochondrial mass as indicated by elevated levels of mitochondrial markers, and in platelets possibly by a post-translational regulation of mitochondrial respiratory capacity. In addition, a plasma factor seems to be able to induce increased uncoupling of respiration in platelets during sepsis.

# BACKGROUND

## MITOCHONDRIA

### Structure and function

Energy is a prerequisite for all life on earth. Plants utilize the energy provided by the sun for maintenance, growth and reproduction in what is called photosynthesis. The carbohydrates, fat and proteins generated in this process can then be used, in a reversed manner, by eukaryotic cells without photosynthesis to release and utilize the energy stored in these substrates. This process is called respiration and can be divided in some distinctly separated steps. For carbohydrates, the first step is the breakdown of glucose (and other sugars) to the three carbon molecule pyruvate. This process occurs in the cytoplasm of the cell. Pyruvate is then actively transferred into the mitochondria where the subsequent steps ensue.

Structurally, mitochondria consist of four compartments: the central matrix, the outer and inner membrane and the intermembrane space. The two membranes are made up of lipid bilayers with great difference in their properties. Whereas the outer membrane is quite permeable to molecules and solutes due to a large number of pores, the inner membrane is impermeable to water-soluble molecules and ions and any exchange over the membrane has to be carried out by specific transporters. In response to different stimuli the inner membrane also has the possibility to increase its area by folding, creating the so-called mitochondrial cristae.

Even though the mitochondrion's primary function is the production of bioenergy it is, among other things, also involved in the processes of apoptosis,  $\text{Ca}^{2+}$  and  $\text{H}^+$  homeostasis and thermoregulation.

Within the central matrix, the second phase of respiration takes place as pyruvate is oxidized to acetyl-CoA, which enters the Krebs tricarboxylic acid (TCA) cycle (first described by Hans Krebs in 1937 [1] and which rendered him the Nobel Prize in 1953). Via further oxidation to carbon dioxide the nicotinamide adenine dinucleotide  $\text{NAD}^+$  and flavin adenine dinucleotide FAD are reduced to NADH and  $\text{FADH}_2$  which then serve as substrates to feed electrons in to the mitochondrial electron transport system (ETS).

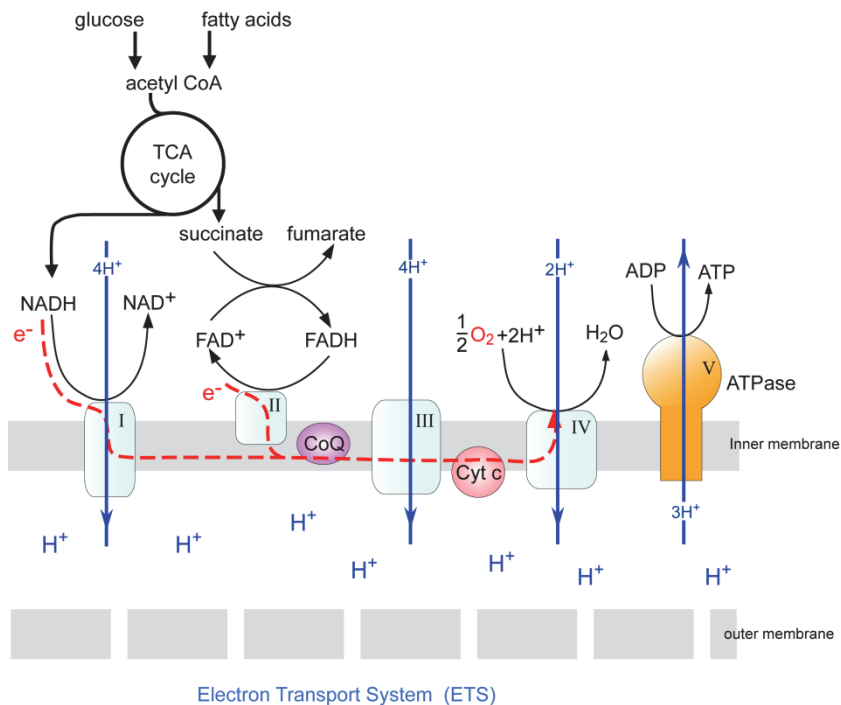


Fig.1 Schematic picture of ATP production by the mitochondrial respiratory system. Reproduced with permission from S. Morota.

The ETS consists of four multiple subunit enzyme complexes bound to the inner mitochondrial membrane. Electrons from NADH are transferred to NADH-ubiquinone oxidoreductase (complex I) and from FADH<sub>2</sub> to succinate-ubiquinone oxidoreductase (complex II). From here, the electrons are further transferred down a redox gradient to the lipid soluble intermediate, ubiquinone (CoQ) which subsequently reduces ubiquinone-cytochrome *c* oxidoreductase (complex III). From complex III the electrons are transferred to cytochrome *c* oxidase (complex IV) via a second intermediate, cytochrome *c* (Cyt *c*). As a last step, complex IV transfers the electrons to oxygen which is then reduced to water. As the electrons are transported down the redox potential, energy is released which is utilized by complex I, II and IV to translocate protons from the matrix to the intermembrane space. By doing so an electrochemical proton gradient is created constituting the so-called protonmotive force. By letting the protons flow back in a controlled manner the energy released is utilized by F<sub>1</sub>F<sub>0</sub> ATP synthase (complex V). Complex V works as a rotary motor to force the phosphorylation of ADP to ATP thereby creating a high energy phosphate bond. ATP is then exported to the cytosol in exchange for ADP by the adenine nucleotide translocator (ANT) to be used in the energy requiring processes throughout the body [2]. The coupling of the energy yielded by oxidation to that of phosphorylation of ADP to ATP was discovered in 1961 by Peter Mitchell and

rendered him the Nobel Prize in 1978 [3]. A schematic figure of the ETS and the phosphorylating system is depicted in Fig. 1.

Until recently, the ETS complexes were viewed as separate units moving freely within the inner mitochondrial membrane where the electrons flow between them and the mobile carriers CoQ and Cyt *c* by random collision. By a more gentle preparation and visualization process (blue native gel electrophoresis) this random diffusion model has been challenged. Using this technique, the complexes appear to be assembled into supramolecular structures called supercomplexes or respirasomes [4-6]. The respirasomes can have different compositions of complex I, III and IV but complex II has so far not been associated with any other complex [6]. The functional significance of the respirasome is still obscure. The proposed benefit of enhanced electron flow due to increased proximity between complexes and CoQ and Cyt *c* still remains to be proven [7,8].

It's essential that the inner membrane is as highly impermeable to solutes and ions as possible as it otherwise would be impossible to create the protonmotive force. However, no membrane is 100% impermeable and there is always a small fraction of the protons that are able to leak back into the matrix, bypassing complex V [9]. This slip lowers the chemiosmotic potential over the inner membrane which in turn stimulates the ETS to increase the transport of electrons and extrusion of protons as compensation [10,11]. Since this ETS activity is not connected (coupled) to the process of making ATP it is denoted uncoupled respiration and is a natural occurring phenomenon that can account for up to 20-25% of normal mitochondrial respiration and hence of the basal human metabolic activity [12]. The proton leak is in small part mediated by the membrane lipid bilayer itself [13] but the major part of basal proton conductance correlates with the levels of the mitochondrial anion carrier family (MACF [14]). The MACFs constitute a family of trans-membrane proteins that are necessary for the transport of metabolites across the membranes of the different cellular organelles [15,16]. Since most of these transport processes are energy-dependent the translocators utilize the protonmotive force directly or indirectly.

Some special members of the MACF are the so-called uncoupling proteins (UCPs) of which UCP1, 2 and 3 are the most investigated [17,18] but also UCP4 and 5 have been described [19,20]. These are inducible proteins that can be upregulated when in need of increased (non-ATP generating) proton conductance over the inner mitochondrial membrane. UCP1 is mainly expressed in brown fat tissue [21,22]. UCP2 is expressed in various amounts throughout the body while UCP3 is mainly found in skeletal muscle and the heart and UCP4 and 5 are predominantly found in the brain [23].

The energy derived from uncoupled respiration is mainly released as heat. Regulation of thermogenesis is therefore suggested as the main function of uncoupling, especially by UCP1 in brown adipose tissue. But also regulation of energy metabolism, control of body mass and regulation of the production of reactive oxygen species (ROS) have



been implicated [23]. As a consequence of being able to attenuate ROS production and thereby reducing the damage caused by these compounds, especially on DNA, one of the main functions of UCPs could be to play a role in the anti-oxidative defense of the cell. By lowering the protonmotive force across the inner mitochondrial membrane the pressure of electrons to exit from the ETS and form free radicals is reduced [24,25]. In situations of increased ROS production, such as various types of stress and diseases, UCPs could thus serve as inducible preventers of excess free radical production [23]. This is also supported by the fact that UCPs are induced by ROS itself [26]. This putative mechanism has also been implicated to be involved in the regulation of senescence, protecting the cells and organs from ageing [27].

## Regulation

At fertilization, mitochondria are transmitted from the oocyte's cytoplasm and are thus strictly maternally inherited [28]. Then, during a normal cell cycle mitochondrial mass increase continuously and is subsequently equally divided to the new cells at mitosis [29,30].

The term "mitochondrial biogenesis" is often used to describe the cellular response to increased mitochondrial mass, both during the normal cell cycle as well as the response to various external stimuli. The main hypothesis of how mitochondrial biogenesis occurs can be divided into three main theories; (i) *de novo* synthesis of mitochondria from submicroscopic precursors present in the cytoplasm; (ii) formation from other membranous structures of the cell; (iii) growth and division of pre-existing mitochondria. The latter is generally the most favored concept [31].

Mitochondrial biogenesis is subject to complex physiological control. Each mitochondrion contains 2-10 copies of mitochondrial DNA (mtDNA) which is a circular double strand DNA molecule [32,33]. MtDNA encodes only 13 of the essential respiratory complex subunits (out of the approximately 78 in total) together with the 12S and 16S ribosomal RNAs and 22 transfer RNAs required for mitochondrial protein synthesis [34]. Hence, mitochondrial biogenesis is dependent on protein synthesis derived from transcription and replication of both mitochondrial and nuclear DNA (nDNA) [34].

A number of regulatory proteins that control the transcription of nDNA and mtDNA genes involved in mitochondrial biogenesis have been identified [35]. For the nuclear genes, nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2) have been demonstrated as key transcriptional regulators [36,37] and for the mitochondrial genome mitochondrial transcription factor A (TFAM) [38]. To help coordinate the expression of genes and co-activation of the transcription factors, PGC-1 $\alpha$  (peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) co-activator 1- $\alpha$ ) has

been put forward as the master regulatory protein for mitochondrial biogenesis [39]. PGC-1 $\alpha$  also seems to serve as the link between external stimuli and the induction of biogenesis by being modulated, through cellular signaling systems, by a variety of stimuli such as cold, fasting, exercise and inflammation [40,41]. Recently PGC-1 $\beta$  has also been identified which appear to hold the same regulatory properties as its  $\alpha$ -moiety [42,43].

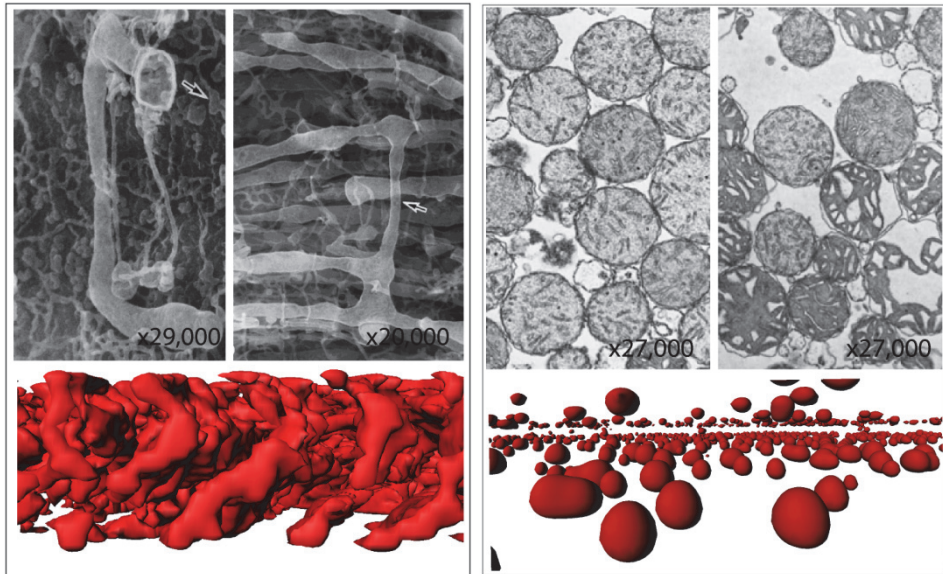


Fig. 2 Electron micrographs (top) and three-dimensional reconstructions from MitoTracker-labelled confocal imaging (bottom) of muscle mitochondria from permeabilized myofibrils (left) and isolated mitochondria (right). Reproduced with permission from [44].

Previously, mitochondria were much thought of as isolated, bean shaped structures within the cell cytoplasm. Whereas this still holds true in some cell types we know now that in most cells the structure and shape of the mitochondria are rather that of a highly branched network that is also very dynamic, changing length and shape throughout the cell cycle and as a response to metabolic demands and external stimuli [45-47]. The processes where the mitochondrial elongates and shortens are governed by the two opposing events, fusion and fission, that are modulated by various mitochondria-associated proteins or by energy substrates [48,49]. The central regulators of fusion and fission consist of several GTPases. Mitofusins 1 and 2 are situated on, and involved in, the fusion of the outer mitochondrial membrane whereas another GTPase, OPA1, localized in the intermembrane space is responsible for the fusion of the inner membrane [50-53]. Another GTPase, Drp1 has to be recruited from the cytosol and acts together with Fis1 (fission protein 1 homolog) to carry out the fission process [45,54,55].

This morphological machinery also seems to be associated with mitochondrial metabolism as a fusion defect results in fragmented mitochondria with reduced ability of respiration and upregulated fusion proteins leads to increased respiration [56-58].

The total mass of mitochondria within a cell is not only governed by biogenesis but includes whole mitochondrial turnover where also mitochondrial degradation plays a role. The term autophagy denotes the process when parts of the cellular membrane and membrane-containing organelles are cleared by the cell and thus, the process of clearing mitochondria has been named mitophagy [59,60]. Mitophagy is thought to play a crucial role in the quality control of the mitochondria within a cell thereby regulating cellular function and resistance to injury [61,62].

## SEPSIS

### Definition

Sepsis is usually said not to be a disease in itself but rather a syndrome defined as the body's response to a non-contained infection. When a pathogen such as bacteria or virus enters the body a localized inflammation emerges initiated by the immune system. Inflammation is a reaction classically defined as "tumor", swelling, "rubor", reddening, "calor", heat, "dolor", pain and "functio laesa", disability. One of the primary goals of inflammation is to recruit immune cells such as neutrophils and lymphocytes and facilitate their ability to enter the infected area from the blood stream. This is achieved by dilatation and increased permeability of the capillaries to increase the blood flow and "leakiness" though the affected area. If the inoculum is too great or the host defense, for some reason, is too weak the infection can spread systemically. This whole body inflammation, which is accompanied by systemic clinical signs such as fever, tachycardia and tachypnea together with an additional array of disturbances, is what we refer to as the clinical condition sepsis.

However, infection is not the only stimuli that can trigger this response but also situations with severe tissue injury such as severe trauma, burns, ischemia and reperfusion injuries, pancreatitis etc. can accomplish the same condition [63]. Even though the term sepsis can be dated to pre-Christian time [64], it was not until 1992 that a consensus definition of the syndrome was reached for the first time [65]. Adjunct to the sepsis definitions the terms systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) were put forward. SIRS was defined as the clinical response to the systemic activation of inflammation regardless of the cause and MODS as the failure of organ function of various degree due to SIRS [66].

The definitions agreed upon were as follows:

For SIRS two or more of the below criteria should be met:

Temperature > 38°C or < 36°C

Heart rate > 90 beats/min

Respiratory rate > 20 breaths/min, or pCO<sub>2</sub> < 4.2 kPa

White blood cell count > 12 x 10<sup>9</sup>/L or < 4 x 10<sup>9</sup>/L or > 10% immature band forms

Sepsis = SIRS as the result of an infection.

Severe sepsis = Sepsis together with organ dysfunction, hypoperfusion or hypotension.

Septic shock = Sepsis with persistent hypotension despite “adequate” fluid resuscitation.

The definitions have been criticized of being too broad and non-specific and an attempt to revise the definitions was made 2001. At this new consensus conference the 4 criteria of SIRS listed above was supplemented with a longer list of clinical signs and symptoms that can be associated with sepsis [67]. Even though none of the new included signs are more specific to sepsis than the previous it expands the possibility for the clinician to suspect and diagnose sepsis. Apart from the expanded list, the definition of the different stages of sepsis remained unaltered [67]. Despite these advances, the definitions are still considered quite vague and a more precise characterization of the syndrome would be beneficial. Lately, translational systems biology derived from computational modeling has been suggested as a way forward to understand the complex and dynamic biological response and help define sepsis in a more precise fashion [68,69]. With regard to diagnosing sepsis, different biomarkers such as C-reactive protein, procalcitonin, various cytokines and markers of organ damage such as neutrophil gelatinase-associated lipocalin (NGAL) have been examined alone or in combination for both the diagnosis and prognostication of sepsis [70,71]. Generally, a combination of biomarkers seems to produce a higher sensitivity and specificity compared to individual markers [72]. Since many of these markers still have to be analysed in research laboratories they have so far not entered clinical praxis. However, with the rapid development of efficient and compact analysis instruments a bedside tool may well be not too far away [73].

## Incidence

The most recent studies on the incidence of sepsis have stated numbers of 0.38 – 0.77 per 1,000 of the population and this is somewhat lower than previously reported, possibly due to different entry criteria to the studies [74-78]. However, with an ageing population and more patients receiving chemotherapy and immunosuppressive

therapy the incidence is expected to rise [76,78]. These figures are all estimates from western countries and no reliable figures are available from the developing countries where the incidence is without doubt higher [79]. Sepsis was not included as an entity in the original Global Burden of Disease (GBD) study [80]. However, with the increasing awareness that the incidence of sepsis has been underestimated and of the global problems, in terms of the morbidity and mortality that it causes, maternal and neonatal sepsis were now included in the new GBD 2010 study [79,81]. Here, neonatal sepsis ranks as number 11 for global causes of years of life lost. Figures on mortality also vary depending on the setting and the severity of sepsis. For severe sepsis mortality has been reported to be approximately 30% in incidence studies [74,75]. For septic shock 28-day mortality was reported as high as 60% in an interventional trial from 2002 [82] but mortality figures have since continuously been reported lower with 40% in two large randomized controlled studies [83,84] and only 24% in a very recent study [85]. There are currently no existing specific therapies for sepsis. Numerous trials of compounds showing promising results in animal studies or phase II clinical trials have failed to prove their benefit in larger randomized controlled trials [86]. Activated protein C did show a survival benefit in an initial study but since subsequent follow-ups failed to confirm this effect it has now been withdrawn from the market [85,87].

## Pathophysiology

### *Immune response*

When the body is damaged, either by an invasive infection or a trauma the innate immune response is stimulated to initiate the inflammatory cascade. Cells of the innate immune system, such as macrophages and dendritic cells recognize a variety of molecules that they associate with “danger”. These danger- or damage-associated molecular patterns (DAMPs) includes both conserved structures derived from invading microorganisms, called pathogen-associated molecular patterns (PAMPs) as well as alarmins, which are molecules released by threatened or damaged cells such as in trauma, burns, pancreatitis etc. [88]. PAMPs consist of evolutionary highly conserved structures such as lipopolysaccharides (LPS) on the gram-negative bacteria and peptidoglycans from the gram-positive bacteria and different molecules presented by viruses, fungi and protozoa [89]. Examples of alarmins are heat shock proteins, fibrinogen, hyaluronic acid and high-mobility group box-1 protein (HMGB-1) [88,90]. These molecular patterns are recognized by pattern recognition receptors (PRRs) which are a set of extracellular and intracellular receptors which can broadly be divided into four categories. The Toll-like receptors (TLR) constitute a family of membrane-bound receptors that was first described in the *Drosophila* [91] and up

until now 10 different receptors have been found in humans where all have been associated with different PAMPs [89,92]. For instance, TLR-4 has been found to recognize and bind to LPS on gram negative bacteria and TLR-2 to peptidoglycan from gram positive bacteria [93]. The receptor for advanced glycation end-products (RAGE) is another membrane-bound receptor that is, amongst others, known to be activated by HMGB-1. For recognition of intracellular pathogens the retinoid acid-inducible gene I (RIG-I) receptors (RLRs) and nucleotide-binding domain, leucine-rich repeat proteins (NLRs) are described as being the most important [89,94,95].

When the PRRs are triggered, a complex event of intracellular signal transduction, including different adaptor proteins such as MyD88, MAL, TRIF and TRAM are activated resulting in a cascade of phosphorylation involving multiple protein kinases. This ultimately leads to the activation and nuclear translocation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) which is a transcription factor known to regulate more than 200 genes involved in inflammation, apoptosis and cell proliferation [96,97]. Essential to inflammation is the upregulation of cytokine and chemokine production. These are small molecules acting as mediators of stimulatory and inhibitory signals in an autocrine and paracrine manner on cells in close vicinity to each other and their production rise tremendously during sepsis and inflammation [92]. The main pro-inflammatory cytokines are regarded as Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) and the interleukins, IL-1 $\beta$  and IL-6 but a multitude of other factors such as interferon- $\gamma$  (INF- $\gamma$ ), macrophage migration inhibitory factor (MIF), HMGB-1 and IL-8, 12, 17 and 18 are suggested to participate in the pathogenesis of sepsis [90,97]. Almost simultaneously there is an activation of a set of cytokines that act as anti-inflammatory. The mounting of this response is important as it acts to counterbalance the pro-inflammatory cascade. IL-4 and 10 are regarded as the two main cytokines but others include TNF- $\beta$ , IL-1ra and sTNFr. The recognition that both pro- and anti-inflammatory activities are initiated simultaneously has emerged over the last decades and has been termed the compensatory anti-inflammatory response syndrome (CARS) [98]. Earlier, sepsis was just thought of as an excess pro-inflammatory response and patients succumbed when they were not able to resolve the inflammation. There have been several clinical trials where interventions were aimed at blocking pro-inflammatory cytokines where none have reliably shown any benefit in survival [99-102]. Recently, a trial of an anti-Toll-like receptor-4 also failed to demonstrate any improvement in 28-day mortality [103]. Studies on septic mice that were either TLR-4 knockouts or where the TLR signaling pathways have been blocked pharmacologically have demonstrated increased mortality [104,105]. Evidently, and in retrospect maybe not surprising, the evolutionary concept of being able to mount a pro-inflammatory response is necessary for recognition and clearance of the invasive infection and therefore ultimately for survival.

Lately it has been suggested that patients that become critically ill for a longer period of time (more than 2-3 days) instead enter a protracted state where the anti-inflammatory response is dominant [106]. In a post-mortem study, it was

demonstrated that patients that died from sepsis exhibited marked reduction of immunological cells of both the innate and adaptive immune system [107]. Also, it has been reported that both pro- and anti-inflammatory cytokine production is markedly reduced in immune cells retrieved from critically ill patients and stimulated with LPS [108-110]. As a consequence, this could render the patient more susceptible to secondary infections with the potential of worsen outcome. It has therefore been hypothesized that immunostimulation would be beneficial to critically ill patients. However, a study of granulocyte colony-stimulating factor in patients with severe sepsis did not show any survival benefit [111]. Also, INF- $\gamma$  treatment in trauma patients did not prevent infection or affect overall mortality [112]. Ideally, it seems that the SIRS and CARS phases should be coordinated in a delicate manner to abate the invading infection without letting any side get out of control.

### *Intravascular coagulation*

During sepsis the coagulation cascade is also activated and can in its extreme form develop to disseminated intravascular coagulation (DIC) where micro-thrombi are generated in the capillary network obstructing blood flow [113]. At the same time coagulation factors and platelets are consumed to such extent that the patient is simultaneously hypo-coagulating with an increased risk of bleeding. Worsening of coagulopathy over the first days of sepsis has also been shown to be associated with increased mortality [114]. Perhaps the most potent instigator of the coagulation system is tissue factor (TF). This protein resides on cells in close vicinity to the circulation enabling coagulation should the endothelial barrier be broken [115] and forms a complex with factor VII of the coagulation system which initiates further cleavage and activation of downstream coagulation factors. In sepsis, mononuclear cells that are stimulated by cytokines are also able to express TF [116] and it has been suggested that TF circulates freely in blood during sepsis [117,118]. The generated products of the activated coagulation such as thrombin are also able to further stimulate the inflammatory response thereby creating a self-sustaining circle. Phylogenetically the activation of coagulation in inflammation has been an essential defense mechanism in order to contain the infection and minimizing the risk of systemic propagation where a fibrin network can act as a trap for bacteria [119]. Microorganisms have in turn developed defense- or propagation mechanisms enabling them to lyse the fibrin clots [120,121]. As with the inflammatory response the coagulation system has its natural anti-coagulant system which is modulated by three major proteins; tissue factor pathway inhibitor (TFPI), anti-thrombin III (AT-III) and the protein C/protein S mechanism. In sepsis, there is a downregulation of these proteins further enhancing the pro-coagulant state [122,123]. With the central role of coagulation, being able to regulate also the inflammatory response, it was believed that supplementing and enhancing the anti-coagulation pathway could be beneficial in the treatment of sepsis. As discussed above, the initial trial of activated

protein C (Xigris®) showed promising results with decreased mortality in the most severely ill patients (APACHE II score > 25) but due to lack of confirmatory studies it is no longer in use [85,87]. Neither have supplementation of AT-III or TFPI demonstrated any survival benefits [124,125].

Platelets are an essential part of clot formation in the blood. They become activated by exposure to collagen at vascular rupture but can also be activated by cytokines. The surface of the activated platelet serves a catalytic environment for the coagulation cascade to occur, resulting in formation of thrombin which mediates positive feedback loops enabling further activation of platelets. When activated, platelets excrete P-selectin thus interacting with neutrophils (see below) and can also contribute to further escalation of inflammation by secreting pro-inflammatory proteins. A fairly recent discovery is that platelets express TLR-4 on their surface suggesting that they can become directly activated by bacteria [126]. Another role of platelet TLR-4 seems to be the facilitation of neutrophil extracellular traps (NET) formation. Neutrophils that are activated by bacteria can extrude their nuclear DNA where decondensated chromatin forms a web-like structure that can ensnare bacteria. This event primarily takes place in the liver sinusoids and pulmonary capillaries through which the blood is filtered. The NET also contains proteolytic activity which facilitates killing of the bacteria [127]. This process often takes 2-4 h but can be shortened down to minutes by activated platelets thereby giving platelets a role not only in coagulation but also in the immune system [128].

### *Endothelium*

In close relation to the coagulation system is the role of the endothelium. The endothelium lines all vessels of the circulatory system and serves as the barrier between the blood and organs and has been proposed to play a crucial role in the septic syndrome and development of MODS. The endothelial cells can become activated by virtually all components of the inflammatory system, *i.e.* cytokines, activated coagulation proteins, activated platelets, the complement system etc. They also possess TLRs whereby bacterial components can induce direct stimulation [129,130].

Upon activation, endothelial cells undergo changes such as swelling and increased permeability with increased extravasation of fluid as a consequence. They can detach from the basement membrane and free endothelial cells have been found in the circulation of septic patients [131]. Adhesion molecules, P- and E-selectin, ICAM-1 and VCAM-1 are expressed on the cell surface which enables rolling and adhesion of circulating neutrophils facilitating their exit to the infected area [132,133]. They contribute to the pro-coagulant state by alterations of their cell surface and interactions between leukocytes and platelets [134].



Again, these changes derive from a natural and evolutionary purposeful response where the aim is to enhance the clearance of invading microorganisms. The upregulated adhesion molecules, increased vascular permeability and creation of edema in the connective tissue facilitate the migration of leukocytes and other immune cells to the site of infection. The pathophysiological role of the endothelium is further supported by results from a septic mice model where the endothelial cells could not express NF- $\kappa$ B. In these knock-out mice, sepsis and MODS were attenuated and which resulted in improved survival compared to wild-type mice [135].

### *Nitric Oxide*

Regulation of vasomotor tone and thereby regulation of systemic blood pressure is partly under endothelial control. Endothelial cells produce vasoactive molecules which include nitric oxide (NO) and prostacyclin as vasodilators and endothelin and thromboxane A<sub>2</sub> as vasoconstrictors. Under normal conditions NO is produced in low concentrations by nitric oxide synthase (NOS) which exists in two isoforms, endothelial (eNOS) and neuronal (nNOS). A third constitutive isoform, residing on the inner mitochondrial membrane, has also been proposed but is still debatable [136,137]. In sepsis there is an increased production of NO due to stimulation by cytokines and other inflammatory mediators of the inducible endothelial nitric oxide synthase (iNOS) [138]. The overproduction of NO is a major contributor to the development of septic shock where it causes depressed contractility of vascular smooth muscle cells together with an increased resistance to catecholamines [139,140]. Inhibiting NOS has been shown to restore the hemodynamics in septic shock. However, in a large clinical trial in patients with septic shock mortality was higher in the intervention group at an interim analysis and the trial was stopped early, underscoring once again the difficulty of intervening in a balance/counterbalance regulated system [141].

Genetic polymorphism is also being recognized to play a role in the development of sepsis and subsequent outcome. Many studies have shown that variations in genes encoding for proteins of the innate immune system can alter the susceptibility and response to infection [142,143]. That these variations also can be inherited was nicely illustrated in a study in adoptees where it was shown that if the biological parent died premature of an infection the adoptee children were also at increased risk of premature infection-related death [144].

## Mitochondrial function in sepsis

Even though there are no specific treatments, algorithms and specific care bundles have been put together to form recommendations for the management of sepsis. Their main features are aiming at prompt infectious control with initiation of antibiotic treatment and source control and early fluid resuscitation in order to reverse the circulatory hypodynamic shock-phase and restore tissue oxygen delivery [145,146]. A successfully resuscitated septic patient enters a hyperdynamic phase which is characterized by high cardiac output, low vascular resistance and elevated oxygen content of the returning venous blood. Even though there is a clear survival benefit in this initial rise in oxygen delivery [146], further increase to supranormal values has not proven to be beneficial [147] and could even be harmful [148]. The high oxygen venous return and the fact that several studies indicate that tissue oxygenation remains normal or even elevated [149-151] during sepsis are thus indicative of a depressed utilisation of oxygen by the cells. Mitochondria are responsible for about 90% of the body's oxygen utilization [152] and altered function of oxygen hemostasis therefore implies involvement of this organelle. The term "cytopathic hypoxia" was coined for this pathophysiological state where decreased mitochondrial respiration and consequently ATP production despite normal or elevated oxygen tension were proposed as the mechanism for organ dysfunction during sepsis [153].

The exact role of how mitochondria is inhibited or damaged is still not clearly elucidated but there are several mechanisms postulated which can act alone or in synergy with each other [154,155].

Pyruvate dehydrogenase (PDH) is the link between glycolysis and the TCA cycle and is considered as the main regulator of glucose oxidation. It is a multienzyme complex that oxidizes pyruvate in the presence of coenzyme A (CoA) to form acetyl-CoA which then enters as substrate in Krebs cycle. PDH has been found downregulated in sepsis thereby diverting glycolysis to lactate formation and inhibiting oxidative phosphorylation by substrate depletion [156].

NO can inhibit mitochondrial respiration directly by binding to cytochrome oxidase (complex IV) where it competes with oxygen at its binding site and thereby impairs the electron flow through the ETS and consequently ATP production [157]. This binding is reversible and under normal conditions, respiration seems to be under a constant reversible NO suppression modulating and matching oxygen consumption [158,159]. Additional damage to mitochondria can be evoked by NO when it combines with reactive oxygen species (ROS).

While the major proportion of electrons that flow through the ETS is transferred to molecular oxygen at complex IV thereby reducing it to water there are electrons that exit this path. As a consequence molecular oxygen may be only partially reduced

creating different ROS such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical (OH). Mitochondria are the major source of ROS production under normal conditions where they have a physiological role, amongst other, serving as intracellular messengers [160]. Due to their reactivity and potential toxicity to mitochondria and other cell structures the production of ROS is tightly controlled by powerful anti-oxidative defense systems which includes manganese superoxide dismutase (MnSOD), reduced glutathione (GSH) and thioredoxin (TSH), peroxidase and catalase [161,162]. If the flow of electrons through the ETS is impaired the complexes and intermediates become increasingly reduced and ROS production increases. In sepsis the production of ROS is greatly enhanced and the anti-oxidant systems become overwhelmed [163,164]. ROS cause damage to a variety of molecules such as lipids, proteins and DNA in both cells and mitochondria and this process is termed oxidative stress [165]. NO in itself is a free radical and reacts easily to produce reactive nitrogen species (RNS). When reacting with  $O_2^-$  it produces peroxynitrite (ONOO<sup>-</sup>) which is a strong oxidizing agent with the capability of causing the same macromolecular damage as ROS. In addition peroxynitrite has been proposed to impair the respiratory complexes I-V and increase leak of protons over the inner mitochondrial membrane [157]. In contrast to the reversible inhibition caused by short-term exposure of NO, inhibition by peroxynitrite tends to be irreversible and is probably due to protein nitrosation. There remains some controversy as to the relevance and mechanisms of complex II and III inhibition [157]. The inhibition of the respiratory complexes can thus stimulate to further ROS production inducing a self-propagating vicious circle. ROS can also activate NF- $\kappa$ B which further stimulates inflammation as described above [166]. There are several anti-oxidant agents that have been suggested as potential therapy in sepsis and that have shown promising results in animal models. So far, however, none has been tested and found beneficial in humans [167].

Another proposed mechanism of peroxynitrite leading to mitochondrial dysfunction is its ability to induce single stranded DNA breaks. This induces a DNA repair mechanism which is conducted by poly(ADP-ribose) polymerase (PARP). PARP uses  $NAD^+$  as a substrate for ADP ribose which in a situation of increased DNA damage can deplete the cell of  $NAD^+$  leaving the oxidative system devoid of reducing equivalents. Regeneration of  $NAD^+$  is energy-dependent further depleting ATP stores of the cell [168,169].

Taken together there seems to be no doubt that several physiological events and molecules produced during sepsis have the potential to affect mitochondrial function negatively with the possibility that this impairment can contribute to the development of organ failure. It has been debated if an eventual metabolic shut down is solely harmful for the organism. An alternative perspective has been put forward suggesting that mitochondrial alterations during sepsis could be a protective strategy where the organs go into hibernation as a consequence of the septic insult. Like winter hibernating animals a reduced metabolic activity would spare the organs of

harm in a state where energy requirements are scarce and better needed elsewhere in the combat of invading organisms [170].



# OBJECTIVES

The main objective of the present thesis was to evaluate mitochondrial function in circulating blood cells in both healthy subjects and in patients with sepsis using high-resolution respirometry. The study specific aims were:

- To implement a methodology for analysing normal platelet respiratory function *ex vivo* in intact viable cells and determine individual complex function in permeabilized cells.
- To assess the impact of storage time and the influence of gender and age on platelet mitochondrial function.
- To analyze blood cell respiration in different reference cohorts in a wide age-span including umbilical cord blood.
- To examine changes in platelet mitochondrial respiratory function during the first week in patients with severe sepsis or septic shock.
- To evaluate the possible role of soluble factors present in plasma on mitochondrial function in sepsis.
- To determine how changes in platelet mitochondrial function in patients with sepsis correlate with clinical parameters, severity of disease and outcome.
- To explore the potential correlation between cytokine and nitric oxide levels in plasma and changes in mitochondrial respiration in sepsis.
- To investigate alterations in mitochondrial respiratory function of peripheral blood immune cells during the first week in patients with severe sepsis or septic shock.



# METHODS

## STUDY POPULATION

The studies were approved by the regional ethical review board of Lund, Sweden, adults: 113/2008, 79/2011, 89/2011 (paper I-IV), and 644/2009 (paper I), children: 59/2009 (paper I) and the ethics committee of Tokyo Medical University, Japan, permit no. 1514 (paper I) and by the scientific ethical committee of Copenhagen county, Denmark, H-C-2008-023 (paper II-IV). For Swedish controls, blood samples were collected from healthy blood donors at the blood donor central, Skåne University Hospital, Lund and healthy adults undergoing rehabilitation after knee injury, medical students and relatives to an unrelated study cohort. The Japanese cohort consisted of healthy adult volunteers. Umbilical cord blood was sampled after delivery from healthy individuals undergoing a normal pregnancy. Samples were obtained after written informed consent was acquired. The pediatric control samples were obtained from patients undergoing minor elective surgery (inguinal hernia repair or phimosis surgery). Written informed consent was acquired from parents or guardian and blood was drawn before induction of anesthesia. Severe sepsis or septic shock was defined as outlined in the background with criteria taken from the 1992 consensus conference [65]. Patients were recruited between August 2008 and September 2011 from the intensive care units (ICU) of Skane University Hospital, Lund, Sweden and Copenhagen University Hospital, Rigshospitalet, Denmark (18 patients for paper II and 20 patients for paper III). Blood samples were taken after written informed consent was acquired from the patient or next of kin. For patients in Denmark deemed temporarily mental incompetent, consent from the patient's primary health care physician was also required. Patients were included within 48 h after their admission to the ICU. Diagnosis of sepsis should have been made no more than 24 h prior to ICU admission. Patients with platelet count  $<10 \times 10^9/L$ , pregnancy, known mitochondrial disease or hematological malignancy were excluded. Blood samples were taken at three different time points during the first week following admission to the ICU; within the first 48 h (day 1-2), on day 3-4 and day 6-7. If a patient received platelet transfusion a minimum of 6 h had to pass before blood sampling.



## Sample acquisition and preparation

A volume of 21-40 ml, from adults, 6-12 ml, from children, and 3-6 ml from umbilical cord was drawn in K<sub>2</sub>EDTA tubes (Vacuette®, Greiner Bio-One GmbH, Kremmünster, Austria). In pilot studies, K<sub>2</sub>EDTA were shown to result in the best yield and prohibit platelet activation compared to Heparin, Citrate and Acid Citrate Dextrose (ACD) as anti-coagulants (data not shown). Blood samples were freshly prepared and analyzed within 3-5 h. For platelets, the tubes were centrifuged 15 min at 300 x *g* in room temperature, to yield a platelet-rich plasma (PRP). This PRP was pipetted off and centrifuged for 5 min at 4600 x *g*, at room temperature, producing a close to cell free plasma and a platelet pellet. The pellet was dissolved in 1-3 ml of the control subject's own plasma by gentle pipetting to obtain a highly enriched PRP with a mean final concentration of 1864 x 10<sup>6</sup> cells/ml (range 941–2498). Peripheral blood immune cells (PBICs) were isolated from whole blood by Ficoll gradient [171] centrifugation. After washing in normal saline, cells were resuspended in 200–400 µl of saline, depending on yield, together with 50–100 µl of the subject's own plasma. Median cell count after preparation was 100 x 10<sup>6</sup> cells/ml (range 15-280). The cell free plasma was frozen and stored until further use.

### *Chemicals*

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) if not stated otherwise.

## HIGH-RESOLUTION RESPIROMETRY

Respiration was measured at a constant temperature of 37°C in a high-resolution oxygraph (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria [172]) in 2 ml glass chambers with stirrer speed 750 rpm. Data was recorded with DatLab software 4.3 (Oroboros Instruments, Innsbruck, Austria) with sampling rate set to 2 s. All experiments were performed at an oxygen concentration in the range of 210–50 µM O<sub>2</sub>. If necessary, reoxygenation was performed by partially raising the chamber stopper for a brief air equilibration. Instrumental background oxygen flux was measured in a separate set of experiments and automatically corrected for in the ensuing experiments according to the manufacturer's instructions. Respiration measurements in permeabilized cells were performed in a mitochondrial respiration medium (MiR05) containing sucrose 110 mM, HEPES 20 mM, taurine 20 mM, K-lactobionate 60 mM, MgCl<sub>2</sub> 3 mM, KH<sub>2</sub>PO<sub>4</sub> 10 mM, EGTA 0.5 mM, BSA 1 g/L, pH 7.1 [172]. Experiments in intact cells were performed with the cells suspended in either phosphate buffered saline (PBS) with addition of 5 mM glucose or in the

control subject's own plasma. The oxygraph was calibrated each day before starting experiments by letting Millipore water or respiration media stir with air in the oxygraph chamber until equilibration and a stable signal was obtained. Oxygen concentration was automatically calculated from barometric pressure and solubility factors that were set to 1.0 for water, 0.92 for MiR05 and PBS glucose and 0.89 for plasma [173].

## Experimental protocol for intact cells

For measuring respiration of intact cells, the cells were suspended in either PBS-glucose or the control subject's own plasma. This allows for the evaluation of integrated respiration with all cellular regulatory mechanisms intact and within plasma, a physiological substrate supply. The experiments were started by letting samples stabilize at a routine respiration state, revealing resting cellular energy demands on oxidative phosphorylation (OXPHOS). To evaluate the contribution of respiration independent of ADP phosphorylation, oligomycin (1  $\mu\text{g}/\text{ml}$ , ATP-synthase inhibitor) was sequentially added inducing LEAK respiration state (also known as oligomycin-induced state 4 respiration). Maximal capacity of the ETS was measured after careful titration of the protonophore carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) until no further increase in respiration was detected (mean concentrations for platelets suspended in PBS 6  $\mu\text{M}$  and plasma 100  $\mu\text{M}$ , for PBICs in plasma 100  $\mu\text{M}$ ). Rotenone (2  $\mu\text{M}$ , complex I [CI] inhibitor) and antimycin-A (1  $\mu\text{g}/\text{ml}$ , complex III [CIII] inhibitor) were then sequentially added to inhibit the ETS providing the residual oxygen consumption which was subtracted from the different respiratory parameters in further analyses. In paper I and II, a second experimental protocol was performed, where ETS capacity was evaluated by direct titration of FCCP after stabilization of routine respiration, followed by the same inhibitors as above. This was done in order to evaluate the influence on maximal respiratory capacity by the inhibition of ATP-synthase. Control ratios were derived from FCCP-stimulated maximal respiration divided by LEAK respiration (ETS/LEAK) and routine respiration (ETS/routine).

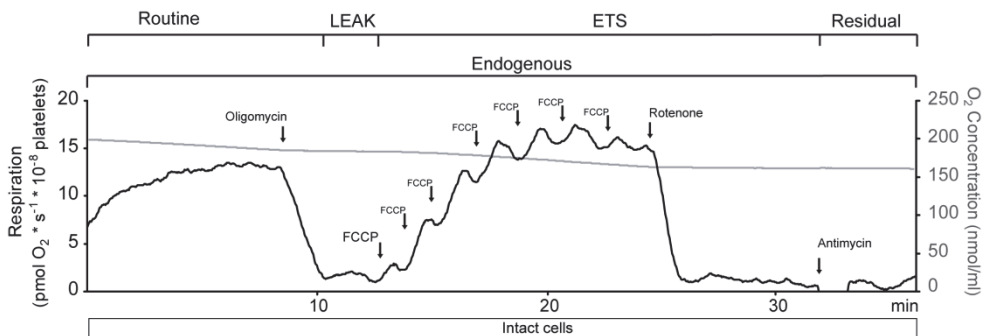


Fig. 3 Representative trace of experiment performed with intact cells.

## Experimental protocol for permeabilized cells

To access the ETS with saturating exogenous substrates and inhibitors the plasma membrane was permeabilized with the detergent digitonin. We determined the optimal concentration of digitonin to induce maximal permeabilization of the plasma membrane without affecting the outer or inner mitochondrial membrane in a separate set of experiments as previously described [174]. Cells were suspended in MiR05 and preincubated with ADP 1 mM, succinate 5 mM and rotenone 1  $\mu\text{M}$ . Digitonin 10  $\mu\text{g}/\mu\text{l}$  was titrated until the maximal response in respiration was obtained. The optimal dosage was found to be 1  $\mu\text{g} / 1 \times 10^6$  platelets and 6  $\mu\text{g} / 1 \times 10^6$  PBICs.

A substrate, uncoupler, inhibitor titration (SUIT) protocol was used to establish the respiratory capacities with electron flow through both complex I and complex II (CII) separately as well as convergent electron input via the so-called Q-junction (CI+II) [175]. After routine respiration was established, titration was started with permeabilization of the plasma membrane with digitonin according to cell type and concentration and a concomitant addition of malate (5 mM) and pyruvate (5 mM). OXPHOS capacity of complex I, driven by NADH-related substrates, was evaluated by adding ADP (1 mM), and additionally glutamate (5 mM) (OXPHOS<sub>CI</sub>, or state 3<sub>CI</sub>). Sequentially, 10 mM succinate was added inducing maximal OXPHOS capacity with convergent input through both complex I and complex II (OXPHOS<sub>CI+II</sub>, or state 3<sub>CI+II</sub>). Oligomycin (1  $\mu\text{g}/\text{ml}$ ) was used to inhibit the ATP synthase and induce LEAK respiration. Maximal convergent respiratory capacity of the ETS was subsequently obtained by titrating FCCP (ETS<sub>CI+II</sub>, mean concentration 6  $\mu\text{M}$  in platelets and 2.5  $\mu\text{M}$  in PBICs). Complex I was inhibited by rotenone (2  $\mu\text{M}$ ) to assess the ETS capacity supported by succinate through complex II only (ETS<sub>CII</sub>). Finally, electron flow through the ETS was inhibited by addition of antimycin-A (1  $\mu\text{g}/\text{ml}$ ), inhibiting complex III, providing the residual oxygen consumption not related to the ETS. After reoxygenation to a level of 160-180  $\mu\text{M}$   $\text{O}_2$ , complex IV

activity was assessed by the addition of adding N,N,N',N'-tertamethyl-p-phenyldiamine (TMPD, 0.5 mM) an electron donor to complex IV. Due to autoxidation of TMPD, sodium azide (10 mM) an inhibitor of complex IV was added and the remaining chemical background was subtracted for calculation of complex IV activity. Control ratios were derived from maximal oxidative respiration or maximal FCCP-stimulated respiration divided by LEAK respiration ( $\text{OXPHOS}_{\text{CI} + \text{II}}/\text{LEAK}$  and  $\text{ETS}_{\text{CI} + \text{II}}/\text{LEAK}$  respectively).

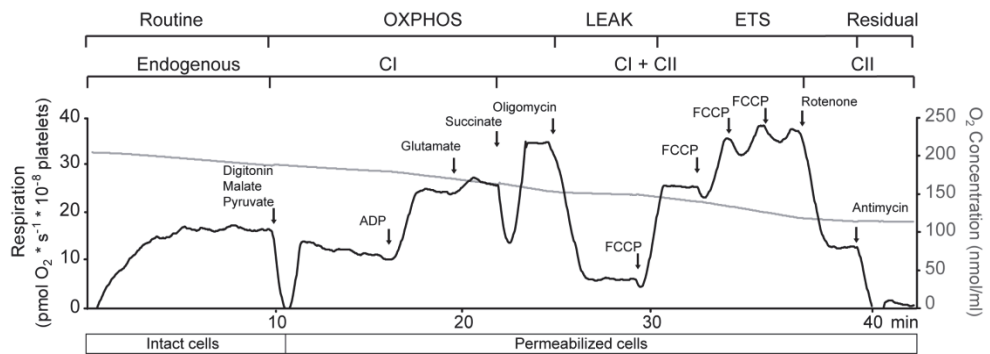


Fig. 4 Representative trace of experiment performed using permeabilized cells. Note, TMPD and azide titration is not displayed due to different scaling.

## Determination of platelet mtDNA content

The analysis of platelet mtDNA content was adapted from [176] with modifications. In paper II, frozen samples were thawed and diluted 500 times in a lysis buffer (10 mM TRIS-HCl, 1 mM EDTA, salmon sperm DNA 1 ng/ $\mu\text{l}$ , pH 8.0). In paper III, samples were additionally sonicated before dilution in lysis buffer. 10  $\mu\text{l}$  of the diluted sample was amplified in a 25  $\mu\text{l}$  PCR reaction containing 1 x Power SYBR<sup>®</sup> Green PCR Master Mix using an ABI Prism 7000 real-time PCR machine (Applied Biosystems Inc., Foster City, CA, USA) and 100 nM of each primer (Eurofins MWG-operon, GmbH, Ebersberg, Germany). The primers targeted the human mitochondrial COX-1 gene (forward: CCC CTG CCA TAA CCC AAT ACC A, reverse: CCA GCA GCT AGG ACT GGG AGA GA). The threshold cycle ( $C_t$ ) values were related to a standard curve using cloned PCR products (kindly provided by P. Schjerling, University of Copenhagen, Denmark). Samples were analyzed in pentaplicates (paper II) or duplicates (paper III).

## Cytochrome *c* determination

Human Cyt *c* content was quantified using an immunoassay kit (DCTC0, Quantikine®, R&D systems, Abingdon, United Kingdom). Frozen samples were thawed and sonicated and subsequently processed according to the manufacturer's instructions.

## Citrate synthase determination

A commercially available kit (Citrate Synthase Assay Kit, CS 0720, Sigma), was used according to the manufacturer's instructions. The assay determines citrate synthase (CS) activity by measuring the color change induced by the conversion of DTNB (5,5'-Dithiobis-(2-nitrobenzoic acid) to TNB (thionitrobenzoic acid), in the presence of acetyl-CoA and oxaloacetate.

## Cytokine measurement

Cytokines were analyzed with a multiplex sandwich immunoassay format with electrochemiluminescence according to manufacturer's instructions (MSD® 96-well Multi-Spot®, Meso Scale Discovery, Gaithersburg, Maryland, USA). In short, 96-well plates pre-coated with capture antibodies for TNF $\alpha$ , MCP-1 (monocyte chemotactic protein-1), INF $\gamma$ , IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-8, IL-10 and IL-17 were incubated with plasma samples for 2 h. Subsequently, detection antibodies were added and the plate incubated for another 2 h. After washing, the plate was read with MSD Sector Imager® (Meso Scale Discovery, Gaithersburg, Maryland, USA).

## NO levels

NO levels in plasma was evaluated by determining its stable metabolites NO<sub>2</sub>+NO<sub>3</sub> using the Griess reaction. A commercial kit was used with modifications adapted for plasma samples [177] (Sigma 23479, nitrate/nitrite Assay Kit Colorimetric, Sigma-Aldrich, St. Louis, MO, USA). Briefly, samples were incubated with nitrate reductase to reduce nitrate to nitrite. Griess reagent was then added and absorbance was measured at 570 nm (Bio-Rad 680 microplate reader, Bio-Rad Laboratories, CA, USA). Background absorbance was measured for each sample and subtracted from total values.

# STATISTICAL ANALYSIS

Data were tested for normal distribution with D'Agostino and Pearson omnibus normality test. Parametric data were presented as mean  $\pm$  SEM and non-parametric data as median  $\pm$  interquartile range (IQR) or range. For parametric data, analysis between two groups was performed using unpaired or paired Student's *t*-test as appropriate. Differences between multiple groups were analyzed with ANOVA with post-analysis using Dunnett's or Tukey's multiple comparison test or unpaired or paired Student's *t*-test with Bonferroni correction for multiple tests. For non-parametric data, analysis between two groups was performed using Mann-Whitney *U* test and for differences between multiple groups Kruskal-Wallis test with Dunn's multiple comparison *post hoc* tests. Correlations were evaluated using linear regression. A p-value of  $< 0.05$  was considered significant. Statistical analysis was performed using GraphPad Prism 5.04 software (GraphPad Software, Inc, La Jolla, CA, USA). For missing values in repeated measurements (paper II,  $n=3$ ) "last value carried forward" was employed.



# RESULTS

Our aim of paper I was first to establish methodology and make thorough assessment of normal platelet respiratory function *ex vivo* in intact viable platelets and of individual complex function in permeabilized cells using high-resolution respirometry. We wanted to evaluate the consistency of the method under various conditions and between different cohorts. We also wanted to collect a reference material to be used not only in the evaluation of mitochondrial function in sepsis but also for the study of patients with neurodegenerative disorders and newborn children with suspected mitochondrial disorders (not described here).

We collected blood samples from 46 healthy adult volunteers (24 from Sweden and 22 from Japan), 28 male and 18 female, with median age 37, and 25 children, 18 male and 7 female, with median age 4, as well as blood from 22 umbilical cords.

## Mitochondrial respiration of intact platelets

In intact cells the respiration is only driven by endogenous substrates or the substrates provided by the incubation medium *i.e.* glucose or plasma-derived substrates in our experimental conditions. We observed that at routine respiration, there was no difference between the two media but after stimulation with FCCP, the spare respiratory capacity, as indicated by the control ratio ETS/Routine, was significantly higher in non-oligomycin treated platelets suspended in plasma compared to PBS glucose. We also observed that maximal respiration after FCCP stimulation was significantly higher in platelets not treated with oligomycin.

## Mitochondrial respiration of permeabilized platelets

In permeabilized cells, both glycolysis and  $\beta$ -oxidation are by-passed by providing saturating amounts of substrates to the citric acid cycle. The result is an ETS and proton circuit that can be evaluated without substrates being rate-limiting. In the presence of malate, pyruvate and glutamate as complex I substrates, ADP stimulation increased respiration by ~90% compared to routine respiration which was similar to the level of maximal FCCP-induced respiration in intact cells. Maximal oxygen consumption, with convergent electron input, either ADP-induced (ATP-generating, OXPHOS<sub>CI+II</sub>) or FCCP-stimulated (non-ATP-generating, ETS<sub>CI+II</sub>), could be



increased by an additional ~50% from respiration with only complex I substrates. The ratio  $\text{OXPHOS}_{\text{CI+II}}/\text{LEAK}$  was ~7.0 and indicated good coupling of electron transport to ATP synthesis and the  $\text{OXPHOS}/\text{ETS}$  ratio was close to one which indicated that almost no flux limitation was exerted by the phosphorylation system at saturating exogenous substrates. In both intact and permeabilized platelets respiration was inhibited by ~99% after complex I inhibition by rotenone and no further decrease in respiration was seen after the addition of antimycin-A or Azide suggesting that platelets use primarily NADH electron input through complex I at physiologic conditions.

There was no difference in respiration between male and female subjects. When plotting different respiratory states against age there was a weak correlation for an increase in routine respiration. We also observed a significant decrease in complex II-linked respiration that was mostly related to a difference between the pediatric and adult cohort.

As there may be limitations in how much blood that can be sampled and how quickly it can be analyzed we wanted to evaluate the limitations posed by storage and small sample volume. We found that in the range of  $100\text{-}400 \times 10^6$  plt /ml respiration remained linear in all states but a concentration of  $50 \times 10^6$  plt /ml maximal FCCP stimulation was decreased by 20-25%. We could also conclude that after up to 24 h of storage in closed EDTA vials there were only minor alterations in respiratory function of the platelets. As a consequence we have performed the majority of ensuing experiments with a platelet concentration of  $200 \times 10^6$  /ml.

## Platelet mitochondrial respiratory capacity in sepsis

After finding out that platelets are well suited for repeated sampling and analysis of mitochondrial respiration we initiated a clinical study that resulted in paper II. Here we examined platelet mitochondrial respiratory function in patients with severe sepsis or septic shock and how it evolved during the first week of the disease. We performed the experiments of intact platelets in the presence or absence of the patients' own plasma which allowed us to address the possible influence of soluble factors affecting respiratory capacity. In permeabilized platelets we could differentiate the contribution of the individual respiratory complexes. We also correlated the changes in mitochondrial respiration with clinical parameters, severity scores and mortality.

We included 18 patients with severe sepsis or septic shock who were sampled three times during the first week; at day 1-2, day 3-4 and day 6-7 and as controls we analyzed 18 healthy blood donors.

We found that platelet mitochondrial respiration gradually increased in septic patients during the first week after admission to the ICU. Depending on experimental

conditions the increase was up to 45% in intact cells and 54% in permeabilized platelets between the first and last time point analysed. Compared to controls respiration had a tendency to be higher already in samples taken within 48 h of admission and by the end of the week the difference was 85% in intact and 67% in permeabilized cells. In order to quantify mitochondrial content in our samples we measured mtDNA and Cyt *c*. The increase seen in mitochondrial respiratory capacity was paralleled by a minor increase in mitochondrial Cyt *c* content in the platelets mainly due to an initial non-significant decrease at day 1-2 compared to controls. The amount of mtDNA did not differ between septic patients and controls and remained at a similar level at the three time points studied. In this first study we suggested that the septic insult stimulated enhanced mitochondrial respiration probably in order to meet the increased metabolic requirements associated with defending the body against the invading organism. The extent of the increase in respiration differed when dividing the septic patients according to 90-day outcome in that non-survivors had a significantly more elevated level of respiratory capacity at day 6-7 compared to survivors. Based on this finding we proposed that the stimuli of enhancing mitochondrial respiration were most pronounced in non-survivors, likely correlating to the severity of the septic insult which we would later examine further in paper IV.

In patients we also observed a difference in LEAK respiration of intact platelets where respiration in platelets suspended in plasma was significantly higher compared to those suspended in PBS glucose. This resulted in a significantly lower respiratory control ratio at day 3-4. This suggested the presence of a soluble plasma factor in the initial stage of sepsis which was able to induce uncoupling of platelet mitochondria leading to a decreased control ratio without inhibition of the respiratory complexes. As free fatty acids (FFA) have been implicated as mitochondrial uncouplers we later analyzed the levels of FFA in the plasma samples from the septic patients. However, the concentration of FFA was similar in patients as compared to controls (results not included in paper II).

## Mitochondrial respiratory capacity in peripheral blood immune cells in sepsis

In the third study of this thesis we moved away from platelets towards the nucleated cells of peripheral blood such as lymphocytes, monocytes and neutrophils. The reasons were severalfold. Firstly, as outlined in the introduction, it is clear that more or less parallel with the pro-inflammatory reaction initiated in sepsis, an anti-inflammatory response is also mounted in order to counter-balance the deleterious effects of a too strong inflammatory response. In this phase immune cells have been suggested to be downregulated leading to an immunoparalysis, or anergy, and mitochondria have been suggested to play a part in this transition. Secondly, since

these are nucleated cells they have the ability to regulate their own mitochondrial mass without relying on whole cell de novo synthesis as is the case with platelets. Also, they reside in the same environment as platelets and are as such exposed to the same stimulatory and inhibitory factors. We were therefore interested in examining any similarities or differences in mitochondrial function of these cells during the course of sepsis compared to our results obtained from platelets.

New patients were recruited with the same inclusion criteria and from the same locations as in paper II, *i.e.* patients with severe sepsis or septic shock that were sampled three times during the first week after admission to the ICU. We included 20 patients and 31 healthy individuals served as controls. PBICs were isolated from whole blood by Ficoll gradient centrifugation. The protocol for analyzing mitochondrial respiration included intact cells suspended in their own plasma and permeabilized cells with the same titration protocol as in paper II with the inclusion of analysis of complex IV. In intact cells, routine respiration was increased compared to controls at day 1-2. Subsequently both routine and maximal FCCP-induced respiration gradually increased so that by the end of the week mitochondrial respiration was approximately 60-70% higher as compared to controls. LEAK respiration was similar to controls on all occasions. In permeabilized cells all respiratory states were significantly elevated already at day 1-2 and had a trend of further increase throughout the subsequent time points. As in paper II, we analyzed Cyt *c* and mtDNA but also added CS as an additional marker for mitochondrial content. At day 6-7 all markers were significantly elevated as compared to controls. The increase in respiration correlated well with the increase of mtDNA number and CS activity and to a lesser extent with Cyt *c*.

Mitochondrial respiration when expressed in relation to CS was significantly reduced as compared to controls at day 1-2 for complex I driven respiration (OXPHOS<sub>CI</sub>) and LEAK respiration at all time points measured. The same trend was exhibited for mtDNA adjusted respiration. Despite this relative reduction in respiration, the control ratios were significantly increased and were similar to those obtained from values of cellular respiration. Our results did not reveal any difference between survivors and non-survivors, in neither respiratory capacity nor markers of mitochondrial content. There were thus similarities between paper II and paper III in that both platelets and PBICs undergo marked increase in cellular mitochondrial respiration during the first week of sepsis. There were also some dissimilarities in that we could not detect any increase in LEAK respiration in intact cells incubated in their own plasma and nor were there any differences in respiratory capacity between survivors and non-survivors.

## Cytokine and nitric oxide expression and their correlation with mitochondrial respiratory function

As discussed in the background, mitochondrial biogenesis can be triggered by several factors that are known to be released in excessive amounts during sepsis. With our findings of increasing mitochondrial respiration in platelets throughout the first week of sepsis and that there seemed to be a correlation between those with the highest respiration and 90-day mortality, we performed a study where we analyzed the plasma content of 10 different cytokines and nitric oxide. We hypothesized that the levels of the cytokines and/or NO in plasma would correlate to platelet mitochondrial respiratory activity. We included the patients from paper II and the 20 patients from paper III whose platelet mitochondrial function also had been analyzed when included in the latter study. The protocols were as outlined in paper II with the additional analysis of complex IV activity. Plasma samples from both cohorts were analyzed for the following cytokines: TNF $\alpha$ , MCP-1, INF $\gamma$  and the interleukins IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-8, IL-10 and IL-17. NO levels in plasma were evaluated by determining its stable metabolites NO $_2$ +NO $_3$  using the Griess reaction. The data from the newly included patients as well as the pooled respiratory data showed a similar increase in respiration throughout the first week of sepsis as the data from paper II. Of the 10 cytokines measured in plasma all, except for INF $\gamma$ , were significantly increased at the first sampled time point compared to controls which were followed by a decline in concentration in the subsequent days analyzed. By the end of the week IL-10, IL-17, IL-6, IL-8, MCP-1 and TNF $\alpha$  remained significantly elevated compared to controls whereas IL-1 $\beta$ , IL-4, IL-5 had returned to values similar to controls. In the septic patients NO $_2$ +NO $_3$  levels were approximately 2.7 times higher as compared to controls but due to large variation the difference did not reach statistical significance. Of the analyzed cytokines, IL-8 levels at day 6-7 correlated positively with both maximal ATP-generating as well as maximal non-ATP-generating rates of respiration *i.e.* OXPHOS<sub>CI+II</sub> and ETS<sub>CI+II</sub>. Also, IL-8 demonstrated the same profile with higher values in non-survivors in samples taken at day 6-7 compared to survivors. There were no correlation between NO levels and mitochondrial respiration or when combining NO and cytokine levels or combining the cytokines most extensively investigated as being pro-inflammatory, namely TNF $\alpha$ , IL-6 and IL-1 $\beta$ . The results thus suggest that cytokines and NO do not play a significant role in the regulation of mitochondrial respiratory function in platelets. Since both CS and mtDNA remained at the same levels throughout the first week after admission the detected increase in mitochondrial respiratory function is more likely related to post-translational regulation rather than increased mitochondrial mass per platelet.



# DISCUSSION

## Assessing mitochondrial function

The main method for analyzing mitochondrial function throughout this thesis has been measurement of mitochondrial respiration using high-resolution respirometry.

Several methods exist to determine mitochondrial function which all have their benefits and drawbacks depending on what questions are being asked and what type of tissue and mitochondrial material that is going to be analyzed. Examples of methods include determination of maximal enzymatic activity of the individual ETS complexes, analysis of respiratory activity in permeabilized cells, intact cells and tissue homogenates and whole body metabolic measurements. Spectrophotometric determination of individual ETS complexes is one of the most common methods used [178]. There are several benefits with this procedure. With its common and wide distribution there is a large reference literature and for most laboratories investigating suspected inherited respiratory chain disorders it remains the method of choice [179]. As for sample handling there are benefits of easy storage and transport to core laboratory facilities with high throughput analyses since the samples can be frozen [179]. Frozen samples have shown acceptable reproducibility for up to 10 years of storage [178].

However, as stated in the introduction, the mitochondrion and its components do not work as isolated units but function in a well regulated physiological milieu interacting with the surroundings. Respiratory chain complexes are interconnected in the ETS that in turn gather to multi-enzyme- and supercomplexes [6]. Mitochondria undergo fusion and fission, form networks and crosstalk with other subcellular compartments [180]. In a recent study, the difference in muscle mitochondrial function was evaluated between permeabilized myofibrils and isolated mitochondria [181]. The authors found a marked difference in isolated mitochondria compared to intact myofibrils, including increased fragmentation of mitochondria, increased sensitivity to mPT opening and increased ROS production. Respiratory capacities were lower in the isolated state for routine respiration but remained similar for state 3 respiration. Further, they observed a selective defect of complex I respiration which they suggest are explained by either (i) loss of soluble Krebs cycle enzymes or intermediates, (ii) proteolytic damage to respiratory complexes, (iii) disruption of supercomplexes during the isolation process. Taken together, this highlights the need to analyse mitochondrial function without, or at least with minimal, cell disruption and with an as close as possible physiological environment. This can be achieved using polarographic measurements of mitochondrial respiration in cells [182].

Respiration can be analysed in intact cells with natural surrounding media, such as plasma, utilizing endogenous substrates. Furthermore, with permeabilization of the cell membrane direct access to the mitochondria for exogenous substrates and inhibitors can be achieved, and individual complexes of the ETS can be investigated without the need for cell disruption and mitochondrial purification [183]. With the refinement of polarography it is now possible to achieve accurate readings with small amounts of tissue sample [184,185].

Since a thorough evaluation of platelet mitochondrial respiratory function, using high-resolution respirometry, had not been evaluated before this was one of our aims in paper I. We developed our protocols in order to obtain as much information as possible well knowing that we in certain patients would have limited amount of sample. It is clear that analysis of intact cells is the condition were the cells are the least affected and by designing one of the protocols to assess intact cells in the patient's own plasma the experimental environment was as close to the *in vivo* situation as possible. However, in order to assess the individual complexes of the ETS it is necessary to permeabilize the plasma cell membrane as most of the mitochondrial substrates used are otherwise unable to reach the mitochondria. The substrate, uncoupler, inhibitor titration protocol thus complemented the assay of intact cells by providing the function of individual units from pyruvate dehydrogenase through complex I–V. Due to initial methodological problems the evaluation of complex IV was not included in paper II.

## Platelets as a representative source of mitochondria

In paper I, II and IV we used platelets as a source of human viable mitochondria. Are they representative for mitochondrial function in other organs? Mitochondrial subunit composition and expression of COX complexes have been demonstrated to vary depending on tissue specific oxidative capacity and/or work load [186] and mitochondrial RNA and proteomics analysis have shown clear tissue-specific differences [187]. Also, diseases affecting mitochondria may be more or less tissue specific. From centers investigating patients for suspected mitochondrial disorders it has been reported that enzyme defects that was found in skeletal muscle only appeared in approximately half of analyzed cultured fibroblasts [178,188,189]. This phenomenon with mixed expression of a mitochondrial pathology is known as heteroplasmy and has as consequence that alterations can go unrecognized if not substantially present in the analysed tissue. Platelet mitochondrial alterations have been demonstrated in a variety of diseases, primarily affecting other organ systems [190,191] as well as in the process of ageing [192,193]. Therefore other authors have suggested that platelet mitochondria could potentially serve as a marker of systemic mitochondrial function [193]. To our knowledge a comparative study of expression

of mitochondrial pathology between muscle and platelet has of yet not been performed. In addition to the studies on septic patients we are currently evaluating the method of analyzing platelet mitochondria as an adjunct to the present standard investigations of suspected mitochondrial disease. We have in this project been able to detect respiratory complex dysfunctions that later have been verified using the regular diagnostic scheme including evaluation of both respiratory and spectrophotometric assays of muscle biopsies (data in preparation).

Evidently the best choice of tissue to investigate for signs of mitochondrial dysfunction is the tissue most profoundly affected by the disease process in a given patient. For sepsis, that would usually mean brain, lung, kidney or heart as these are the organs mostly affected in MODS. However, this is virtually not possible due to the invasiveness and risks associated with biopsies from internal organs such as brain, liver and heart. Thus, until non-invasive techniques are further developed it seems we have to rely on analysis of secondary tissues or animal models. There are still only a handful of human studies in sepsis directly assessing mitochondrial function and in these, muscle is the most commonly used tissue followed by mononuclear cells and platelets [194-200]. Another difficulty when assessing mitochondrial (dys-)function is that cells have the ability to compensate for decreasing levels of ATP and usually exhibit a threshold where compensation can no longer occur and organ failure ensues. This has mostly been described in inherited mitochondrial disorders such as MELAS, LHON and Leigh's disease [201]. However, the same compensatory mechanisms likely exist in other conditions. It is thus difficult to predict at what level an observed decrease in mitochondrial respiration should be considered pathological and contributory to organ failure in sepsis.

## Determination of mitochondrial content

The enzymatic activity of citrate synthase is considered as one of the most reliable indicators for mitochondrial matrix content and is probably the one most commonly used [202,203] followed by the levels of mtDNA [176,204]. Other markers include cardiolipin [205], complex IV activity [181] and total protein content [199]. Larsen *et al.*, investigated, in muscle samples from young healthy volunteers, the relationship between a variety of markers compared to mitochondrial fractional area (MFA) calculated from transmission electron microscopy images, which they defined as the gold standard. Cardiolipin and CS showed the strongest correlation and concordance with MFA. Activity of complex I, II, IV and complex II and V content showed intermediate, while mtDNA demonstrated the weakest correlation. The last finding was somewhat surprising since mtDNA has recently been shown to correlate with CS [206] which was confirmed by our results in paper IV where CS and mtDNA displayed excellent correlation in both the control cohort and the septic patients both



in relation to each other as well as to mitochondrial respiration. In paper II we were not able to measure CS due to technical problems so in that study, only mtDNA and Cyt *c* were used as markers of mitochondrial content.

## Mitochondrial function in sepsis

One of the main findings in this thesis is that in human platelets (paper II) and PBICs (paper III) from patients with severe sepsis or septic shock there is an increase in mitochondrial respiration throughout the week following admission to the ICU. In paper II, we found that the respiratory capacity of platelet mitochondria increased by 29-54% in sepsis depending on experimental conditions and up to about 85% compared to controls and the enhanced respiration was seen in both intact and permeabilized cells. In PBICs the increase in mitochondrial respiration was significantly elevated already at day 1-2 by 30-89% and continued to increase throughout the week by another 21-42%.

However, there was a clear difference between the two cell types that became increasingly apparent throughout the work of this thesis. In platelets the increase in respiration was not accompanied by an increase in mitochondrial mass as indicated by unaltered levels of CS and mtDNA. For Cyt *c* levels, there were an initial decrease of the amount per cell in the septic patients compared to controls which was followed by and subsequent return to similar levels as controls. In the first paper this led us to believe that this could be interpreted as initiation of mitochondrial biogenesis. In paper III we included a larger cohort of patients and also analysed CS as a mitochondrial marker. Since neither mtDNA nor CS differed from controls and these markers had a better correlation to mitochondrial respiration compared to Cyt *c* we reevaluated our conclusions in that the increase seen in platelet mitochondrial respiration does not seem to stem from an increase in mitochondrial mass. It is increasingly recognized that post-translational regulation of the OXPHOS system is an important modulator of respiratory capacity. Complexes of the ETS as well as a majority of the metabolic pathways in the mitochondria have been shown to be subjected to regulation by phosphorylation and dephosphorylation [207]. These intracellular events seem to be, at least in part, controlled by calcium homeostasis and could thus be linked to intracellular signaling cascades, triggered in turn by extracellular receptor interactions [208]. In contrast to platelets, the increase in respiratory capacity of PBICs was accompanied by a similar increase in the markers of mitochondrial content indicating an upregulation of the translational machinery in order to increase mitochondrial mass per cell. It could be speculated that the differences seen could stem from the differences between the two cell types. As anucleated cells, platelets are devoid of translational regulation and cannot respond to an increase in respiratory capacity in other ways than by upregulation in the *de novo*

production process by megakaryocytes in the bone marrow or post-translational regulation. PBICs, on the other hand, have the possibility to generate a biogenesis response thereby augmenting the mitochondrial content within the cell.

Interestingly, most of the events triggering the immune system, *i.e.* bacterial triggering of TLRs and increased ROS and NO production, have all been implicated in the initiation of adaptive mitochondrial biogenesis which is likely triggered to cope with the increasing energy demand required for the recovery from the septic insult [209-214]. Our results are also in line with several other studies. In a recent article, mitochondrial biogenesis was observed in a mouse model of sepsis and survival was higher in the group where biogenesis was further augmented by inhalation treatment of carbon monoxide [215]. Biogenesis has also been proposed to play an important part in the recovery following sepsis. In a murine model of *S. aureus* sepsis, Haden *et al.* demonstrated an early fall (day 1) in liver mtDNA copy number. Subsequently they noted an increase in mRNA levels coding for the biogenesis regulatory proteins NRF-1, NRF-2, TFAM and PGC-1 $\alpha$  already at day 2 after the induction of sepsis. As a plausible consequence, mtDNA copy numbers were restored to normal values at day 3 post-induction of sepsis [212].

It has been argued that the most correct way of determining mitochondrial biogenesis is by assessing mitochondrial protein turnover and that detection of increased markers of mitochondrial content would not be sufficient [216]. This is based on the concept that mitochondrial protein content reflects not only the synthesis but also the breakdown of proteins. Thus, an increased synthesis accompanied by an increased breakdown would result in unaltered content while decreased breakdown with unaltered (or increased) synthesis would result in increased content. The latter should not necessarily be interpreted as positive because it could be a consequence of dysregulated quality control of mitochondria with accumulation of damaged or malfunctioning proteins [216]. Also, using mRNA transcription levels to predict biogenesis could cause erroneous results due to the fact that mRNA upregulation does not necessarily translate to enhanced protein production [216]. Although we agree with the above discussion it is only relevant when these factors are assessed in isolation without also measuring the functional outcome. In both paper II and III our conclusions have been drawn on the basis of content, function and internal normalization using control ratios. By combining these approaches we were able to derive how the increased markers of mitochondrial content resulted in changes in mitochondrial function. To further connect these results to a transcriptional level we also attempted to analyse PGC-1 $\alpha$  protein amount of our PBIC samples in paper III. However, due to the low abundance of the protein, levels were under the reliable detection limit in the majority of samples. We also evaluated a reverse transcript PCR (RT-PCR) protocol in order to analyse the expression of PGC-1 $\alpha$  mRNA. However, since the samples had not been optimized for mRNA detection, which is especially important for samples containing eosinophil leukocytes that contain RNase activity

[217], the amplification was nonspecific and the overall RT-PCR without trustworthy results.

### *Relating changes of mitochondrial content and function*

As observed in paper III the increase seen in mitochondrial respiration of PBICs expressed per cell, changed to a decrease in OXPHOS, at the first time point measured, when related to mitochondrial content. This suggests that within each mitochondrion there was a relative lower increase of some functional units, *e.g.* complex V, as OXPHOS (complex V-dependent) respiration was less elevated compared to CS and mtDNA whereas maximal ETS (complex V-independent) respiration, in relation to the same markers, remained constant. Further, the overall functional integrity of mitochondria in PBICs of septic patients was good as evidenced by a lower LEAK and higher control ratios compared to controls at the different time points studied.

From this it is clear that if the respiratory capacity would have been only expressed as a function of CS the interpretation would have been different. As we could see in our results from paper II and III the kinetics of the mitochondrial markers we had chosen (*i.e.* CS, mtDNA and Cyt *c*) were different and varied between platelets and PBICs. This highlights the necessity of analyzing more than one mitochondrial marker in order to interpret observed alterations in respiratory function.

Several other studies report dysfunctional mitochondrial respiration when related to mitochondrial markers. Brealey *et al.* showed impaired complex I, II and IV enzymatic activity expressed as a ratio of CS activity in septic non-survivors [194]. This was not corroborated in the study by Carré *et al.* where complex I activity tended to be lower in septic non-survivors but remained unchanged when related to CS activity indicating loss of mitochondrial content and not dysfunction *per se* [196]. The same results have emerged from two other studies analyzing muscle mitochondrial function of critically ill patients with MODS [200,218]. Even though there are animal models that have not demonstrated any impairment of mitochondrial function in muscle biopsies [219] there seem to be more consistency in the human studies. Rapid muscle wasting has been demonstrated in sepsis [220] which is probably caused by factors such as increased protein turnover, ROS toxicity and passivity. A decrease in mitochondrial mass in this situation is thus not surprising.

The importance of combined analysis of different mitochondrial respiratory parameters was evident in paper II where platelets displayed both an increase in mitochondrial respiratory capacity and in parallel had a significantly elevated LEAK respiration when incubated in septic plasma, resulting in a decreased control ratio at day 3-4. Increased LEAK respiration and reduced control ratios have been demonstrated previously in sepsis. D'Avila *et al.* found mitochondrial uncoupling in

brain homogenates in a 24 h cecal ligation and puncture (CLP) model in mice [221]. Also, plasma taken from septic patients at day 1 and day 7 induced increased LEAK respiration in peripheral blood mononuclear cells from healthy controls [195]. Uncoupling proteins (UCPs) have been implicated in sepsis where UCP3 has been shown to be upregulated in muscle in a CLP model in rats and UCP2 deficient mice were protected from LPS-induced liver failure [222,223]. Increased LEAK respiration and a lowered respiratory ratio is usually taken as a sign of decreased efficacy of the phosphorylating system but an increase in LEAK has also been suggested to be beneficial since it could potentially reduce ROS production [27]. As ROS production is increased in sepsis the existence of a proton leak-ROS feedback loop has been suggested where ROS increase proton leak which in turn reduces ROS production via lowering of the protonmotive force [23,224]. The mitochondrial permeability transition (mPT) has also been implicated in sepsis [225-227] but cannot readily explain the elevated LEAK respiration in our study. Activation of mPT leads to loss of mitochondrial matrix substrates as well as dissipation of the protonmotive force which uncouples as well as inhibits respiration [228]. However, no inhibition of the ETS was detected in the platelets.

Three other studies have investigated PBICs from septic patients. In the study by Japaissu *et al.* PBICs were investigated using a titration protocol with only complex II-linked substrates. They found that OXPHOS was reduced in the early phase of sepsis as compared to control subjects that constituted critically ill patients without sepsis. No differences were seen in the other respiratory states, *i.e.* routine, LEAK or ETS [197]. In addition, the PBICs of septic patients were, as compared to the controls, more sensitive to oligomycin-induced inhibition. This led the authors to conclude that the impairment of mitochondrial function was due to a reduced content of functional complex V [197]. Also, in the septic survivors, complex II-linked respiration increased 2.9 fold from day 1 to day 7. Our data agree with this latter finding but stand in somewhat contrast with the former because no inhibition of OXPHOS was seen at day 1-2, but rather an increase, of complex II-linked respiration. However, as outlined above we also noted a difference between OXPHOS and ETS suggesting a relatively higher increase in non-ATP-generating respiration (ETS) making complex V, or other part of the phosphorylating system such as ADP/ATP or phosphate translocation, the rate limiting factor in ATP-generating respiration (OXPHOS). The differences seen in OXPHOS capacity between the two studies probably lies in the difference between the controls cohorts. The mean respiration value, with complex II-linked substrates, of PBICs in the critically ill control cohort was approximately double that of the healthy subjects used in the present study. In contrast, the same parameters measured in the septic patients were very similar in the two studies. Since any type of inflammation can potentially trigger mitochondrial biogenesis [41] this possibly implies that respiration was upregulated in the critical ill patients used as controls in the study by Japiassu *et al.* [197].

In the study by Garrabou *et al.* PBICs were isolated from patients with sepsis but without shock. When related to total cell protein content they found decreased enzymatic activity of complex I, II and IV and decreased routine and complex I respiration [199]. They also measured CS as a reflection of mitochondrial content which did not differ between patients and controls. They presented neither values of respiration expressed per cell nor any absolute values of cellular protein content in which however a rise due to a likely activation of PBICs in sepsis is plausible. This makes it difficult to draw any firm conclusion when comparing their findings to our results.

In another study examining mitochondrial respiration in septic patients, PBICs, sampled within 48 h of ICU admission, were found to have elevated routine respiration, most likely due to an increase in LEAK respiration, and inhibited complex II-linked respiration as compared to healthy controls [195]. The authors suggest the presence of a plasma factor that induces uncoupling since healthy cells incubated in septic plasma displayed the same uncoupling as septic cells. Our results on platelets in paper II suggested a similar occurrence of a plasma factor increasing the permeability of the inner mitochondrial membrane. However, LEAK respiration in PBICs did not differ compared to controls in paper III. These differences seen in LEAK and ADP-stimulated respiration is not entirely clear but is possibly explained by the fact that we have measured  $ETS_{CII}$  respiration versus  $OXPHOS_{CII}$  in the study by Belikova *et al.* again suggesting a difference in complex V function. Also we have found that freshly prepared PBICs are prone to aging with consequently relatively rapid deterioration of mitochondrial respiratory function and a role of the longer incubation time in the study by Belikova *et al.* cannot be ruled out.

## Mitochondrial respiration and mortality

In paper II, non-survivors at 90 days displayed significantly elevated levels of respiratory capacity compared to survivors at day 6-7; both in absolute values as well as expressed as control ratios. This finding was not substantiated in the PBICs studied in paper III. Here, we could not detect any difference, between survivors and non-survivors in mitochondrial respiration or in the three different markers of mitochondrial content measured. As the majority of deaths in paper III occurred within the first week as compared to one in paper II, it precludes drawing any firm conclusions regarding these differences found. To our knowledge there is only one other study where platelet mitochondrial function in septic patients has been studied. In this study by Lorente *et al.* [198] 96 patients with sepsis or severe sepsis were included and sampled at the time of diagnosis. The objective was to analyze cytochrome *c* oxidase activity (COXa) and quantity (COXq) in a multi-plate reader combination assay and evaluate any difference between 6-month survivors and non-

survivors. To adjust for mitochondrial content they also measured CS activity and all COX values were expressed as per CS activity. What they found was that COXa/CS and COXq/CS were lower in non-survivors (10% and 43% respectively). CS was also significantly higher in non-survivors by approximately 68%. Since there were no report on the absolute values of COXa and COXq it is not possible to deduce if the difference between survivors and non-survivors stems from an actual decrease of COXa and COXq or if the effect is partially or only due to the increase in CS [198]. Another study looking at transcriptional markers of biogenesis noted that PGC-1 $\alpha$  mRNA were lower in eventual non-survivors and remained similar to controls in survivors [196]. Tissue specific differences could possibly account for some of the discrepancies seen and there is uncertainty regarding to what extent mRNA transcription translates into final functional mitochondrial protein and actual respiration as measured in our studies [216]. Increased mitochondrial respiration is often thought of as beneficial and a requirement or response to the increasing metabolic demands of recovery from sepsis. However, in a situation with enhanced metabolic rate and cellular respiration, oxygen delivery could be mismatched in relation to demand which would subject the tissues to the risk of a relative oxygen deficit. This, in turn, could have negative consequences for cellular and organ function.

## Cytokines

The finding in paper II that non-survivors had higher levels of respiration compared to survivors led us to hypothesize that this could possibly reflect a more pronounced septic insult in those patients which would be reflected in markers of the inflammatory response. Increased plasma cytokine levels have been demonstrated in several studies of human sepsis and both individual cytokines as well as various combinations have been used as predictors for severity of disease and outcome [71,229-233]. TNF $\alpha$ , IL-1 $\beta$ , IL-8 and MCP-1 are all regarded as pro-inflammatory cytokines whereas IL-4 and IL-10 are regarded as anti-inflammatory and IL-6 has been attributed a dual role [90,97]. IL-17 is a recently discovered cytokine that is considered pro-inflammatory in its ability to differentiate the Th-17 T cell lineage [234]. IL-5 is the primary hematopoietic cytokine responsible for eosinophil growth and maturation and has recently been suggested to promote a protective innate immunity response in sepsis [235]. After the initial peak concentration at day 1-2 there was a steady decline at the subsequent time points but IL-10, IL-17, IL-6, IL-8, MCP-1 and TNF $\alpha$  remained elevated throughout the first week which was also found in a large multicenter study investigating the cytokine response in severe sepsis due to pneumonia [71]. In this study they concluded that mortality was highest in the patients with sustained elevated levels of both pro- and anti-inflammatory cytokines. TNF $\alpha$  has been shown to inhibit OXPHOS in liver cells by subunit tyrosine

phosphorylation of complex IV and to cause inhibition of complex I and II in fibrosarcoma cells [236,237]. In contrast, mitochondrial respiration in endothelial cells was enhanced after TNF $\alpha$  incubation and also displayed increased protein content of PGC-1 $\alpha$ , TFAM and NRF-1 as an indication of induced mitochondrial biogenesis [238]. The same phenomenon has also been described for NO where multiple studies have provided evidence for its inhibitory effect of mitochondrial respiration paralleled with its ability to stimulate mitochondrial biogenesis [157,239-241].

In our study we found that IL-8 measured a week after admission to the ICU correlated with mitochondrial respiration measured at the same time point. IL-8 also displayed higher levels in 90-day non-survivors compared to survivors. In accordance with this, IL-8 has previously been analyzed in septic patients where the levels were found to be higher in non-survivors compared to survivors [242,243]. Another study tried to refine the prediction of outcome in septic patients and found that when combining the levels of IL-6, IL-8 and IL-10 they could demonstrate a 2-3 fold increased hazard ratio of not surviving the septic event, both early (day 3) and late (day 28) following admission [230]. This was however not replicated in our study as neither IL-6 nor IL-10, alone or in combination with IL-8, correlated with mortality.

As discussed above, the major part of the increase in respiratory capacity seen in platelets seemed to stem from a post-translational regulation. The mechanisms of this enhancement and the regulatory steps controlling them are not elucidated but could involve a role for cytokines as conveyers of the stimulatory signals. However, our results from paper IV argues against a simplistic direct association between cytokines and NO and mitochondrial respiration. The correlation observed for IL-8 has to be evaluated in further studies in order to make any substantial conclusions.

## Cellular changes

Both platelets and PBICs are cells with a rapid turnover in the body. Under normal conditions platelets circulate approximately 12 days before being removed. The normal circulation for PBICs is more complex as they comprise heterogeneous populations and reside in various reservoir pools such as the spleen from where they are liberated into the bloodstream. All types of PBICs undergo rapid proliferation and differentiation upon activation and turn from small quiescent cells to highly active metabolic effector cells. These changes require large amounts of energy and activation of PBICs has been shown to induce mitochondrial biogenesis [244,245]. Apart from proliferation and cell growth another role linking PBICs and mitochondria in the innate immunity has been proposed as it has been demonstrated that upon stimulation of TLRs, mitochondria are recruited to macrophage phagosomes where

they supply additional ROS generation increasing the macrophages' ability to kill bacteria [246].

Some of the observed increase in respiratory capacity could be related to a high turnover of platelets and PBICs in sepsis creating a more freshly produced pool of mitochondria being studied. The difference in respiratory capacity, if any, of newly produced platelets compared to the circulating pool is not known. As outlined above, the situation with PBICs is even more complicated. In paper III we did not evaluate respiration of subpopulations of PBICs between controls or between the different time points in the septic patients. It is thus not possible to tell if there are any specific subpopulations that alter their mitochondrial function in the course of sepsis. Also, it has been well-established that in sepsis there is a decrease of immune cells especially lymphocytes. This is, however, not necessarily contradictory to our findings of an increased biogenesis response since despite a reduction in total number the turnover of cells is likely to continue. These freshly produced cells, however fewer, would then retain or increase their mitochondrial respiratory capacity in response to the stimuli exerted by the septic process.





# FUTURE PERSPECTIVES AND FINAL CONCLUSIONS

Many studies have shown altered mitochondrial function in sepsis of which several have been referenced throughout this thesis. Most of these demonstrate an impairment of mitochondrial function [194,212,226,247-253], but others have demonstrated improved function [238,254-258] or similar values as controls [219,259-261] and some mixed functional changes [262,263]. Differences in experimental setup and species variation are of course possible explanations and also differences in what tissue that has been analyzed. For laboratory models it has also been proposed that those studies with an experimental setup evaluating mitochondrial function at the very early stages (up to 16 h) have more consistently demonstrated enhanced or no change whereas models spanning over a longer period (days) more consistently have shown reduced function [155]. For the human data that exists, the majority of experiments have been performed at the early phase of sepsis, usually within one or two days after admission to the ICU. This is however usually several days after the patient has fallen ill and the physiological processes started. It is thus very difficult to relate the results from the experimental setup to “real life” sepsis. Also, most patients today survives the initial phase of sepsis, either recovering or entering a more prolonged phase of “chronic” critical illness with sustained multiple organ failure.

Sepsis seems to be a disease of counter-regulatory mechanisms. The pro-inflammatory response is counterbalanced by the anti-inflammatory, the pro-coagulant state with the anti-coagulant. The same physiologic mechanisms also seem to be involved in the regulation of mitochondrial function. It is undoubtedly so that some of the major constituents of sepsis such as NO and some of the pro-inflammatory cytokines can exert inhibitory effects on mitochondria, mostly on the respiratory complexes. However, it is also clear that the same molecules are able to initiate enhanced mitochondrial respiratory capacity. The dual function is also nicely illustrated by quoting two recent articles with different angles on the subject: “The addition of NO donors to cell cultures or intact mitochondria causes a rapid decrease in oxygen consumption...” [155] or “Treatment of various cells with NO donors increases their mtDNA content...” [211].

Summarizing these findings, it thus seems like mitochondrial function in sepsis undergo three phases which are not distinct but have considerable overlap. Firstly, an initial and very early upregulation which probably is mostly post-translationally regulated by phosphorylation, supercomplex assembly etc. Secondly, there is a phase

of impairment mediated by NO, cytokines, FFAs, UCPs etc. This impairment can be due to either a single or a combination of factors including direct inhibition of the OXPHOS system, decreased functional content or increased uncoupling as demonstrated in this thesis. In the third phase there is a trigger of mitochondrial biogenesis and post-translational optimization, not only restoring but also increasing mitochondrial respiratory function to supranormal values.

With these dynamic changes taking place it is not surprising that the results vary to a considerable extent and studies continue to produce conflicting data. Another conclusion that can be drawn from the results of this thesis is the importance of normalization. As described, impaired mitochondrial function can be converted to normal or enhanced function depending on what, how and when markers for mitochondrial content are measured. It is therefore crucial that future studies include multiple markers for mitochondrial content so that the results can be evaluated in a relevant context.

Another perspective that is seldom accounted for is the functional context *i.e.* whether or not any alterations found are of clinical significance. As mentioned above there seems to exist a so-called threshold effect, above which the cells or organs can compensate without expressing any phenotypic alterations [201]. The pediatric diseases in which this phenomenon is mostly described are rather well characterized but despite this, there is still a continuous discussion on how to best diagnose these children and what methods and criteria to use [264]. The introduction of the same discussion in the field of sepsis and MODS would strengthen and facilitate the conduct and interpretation of future research on mitochondrial function. As for now, the truth is that, even though we find some level of mitochondrial impairment or enhancement in a septic patient as compared to controls, we do not know the clinical relevance of this alteration.

Even though some basic data can be derived from analyzing single protein interaction it seems clear that a more holistic and integrative approach has to be embraced if we want to be able to describe the complex physiological and pathophysiological alterations involved in sepsis. In this thesis we have aimed at keeping the experimental procedures as close as possible to the natural physiologic milieu for the cells and mitochondria. With keeping the cells and mitochondria within their natural environment (as close as it is possible *ex vivo*) and analyzing the integrated functions of the OXPHOS and ETS we believe that we have been able to recreate how the mitochondria functions within their natural habitat.

We can however only draw firm conclusions from the tissues that are investigated and this is probably the greatest challenge for the future. As outlined above a human study with samples from the vital organs most profoundly affected in sepsis will probably never take place. As a surrogate there are some non-invasive techniques such as near-infrared spectroscopy that can provide information about the redox state of the mitochondria in some of the tissues not available for biopsy *i.e.* liver, brain and

kidney. These techniques are far from perfect, have low resolution and suffer from some technical and interpretational problems. Whole body magnetic resonance spectroscopy is often not an option in a severely sick, intubated ICU patient even though it has been used in animal studies [265,266].

Much criticism has been raised against the representability of animal models of sepsis applied to humans. The reasons are multiple and most often evident such as differences in species and differences in relative age between animals and humans, where animals tend to be younger. Laboratory animals have no comorbidities as opposed to patients with diabetes, immunosuppression etc. Most animal models use peritonitis as the source of sepsis whereas pneumonia is the most common infection causing sepsis in humans. Patients receive aggressive, individualized treatment with fluids controlled by various monitoring devices, and antibiotics whereas animals sometimes (but not always) receive standardized fluid resuscitation and get no antibiotic treatment. The timing of the insult is known in animals but in humans there is often uncertainty as to when the disease started. Clearly, in order to investigate the underlying pathophysiologic mechanisms in sepsis we must aim at models mimicking human sepsis as closely as possible. This effort has already been pursued in some studies [247] but will have to be developed further especially if we want to elucidate pathophysiologic mechanisms occurring in the later phases of the sepsis and “chronic” critical illness.

As discussed above the alterations of decreased mitochondrial mass found in muscle tissue have so far only been studied in early sepsis or as “point prevalence” in critically ill patients in different stages of their disease [194,200]. Thus it seems logical that the temporal aspects of mitochondrial function in blood cells, found in the papers of this thesis, also should be studied in muscle. With refined microbiopsy technique the invasiveness and risks for the patient can be minimized [267]. In our group we have adopted this technique and evaluated biopsies from vastus lateralis of 20 healthy controls with excellent methodological quality and reproducibility (data not shown). Hopefully a new study can be launched in the future that will include septic patients.

The growing interest and appreciation that mitochondria are involved not only in the inherited diseases but also in many commonly acquired diseases has sparked an intensive research towards developing specific drugs interacting with mitochondrial function. If launched, it would give the opportunity to test the assumption that patients with sepsis and MODS could benefit from altered or enhanced mitochondrial function. However, taken into account the numerous failed trials where a drug has been tested as one intervention modulating only the one side of a delicate counterbalance system *i.e.* inflammation or coagulation, or trials where normal or supernormal values have been the targets makes one humble in regard to the potential performance of such intervention. Likely, a deeper understanding of the role of mitochondria in sepsis and MODS is needed before a long anticipated golden bullet for treatment can be presented.

To conclude, this thesis demonstrates that:

- Platelets are a reliable source of human mitochondria with excellent stability and reproducibility for analyzing mitochondrial respiratory function.
- Platelets and PBICs exhibit increased respiratory capacities throughout the first week of sepsis.
- The increased cellular respiration in sepsis seems to be accomplished by different pathways; in PBICs by increased mitochondrial mass as indicated by elevated levels of mitochondrial markers, and in platelets possibly by a post-translational regulation of mitochondrial respiratory capacity.
- A plasma factor seems to be able to induce increased uncoupling of respiration in platelets in the septic condition.
- Platelet mitochondrial respiration displays higher values after one week in 90-day non-survivors compared to survivors.
- Cytokines and NO do not seem to play a prominent role in the regulation of platelet mitochondrial respiration in sepsis.
- Mitochondrial function has to be related to a variety of specific markers of mitochondrial content in order to place results into relevant context.

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# SVENSK SAMMANFATTNING

Allt liv på jorden är beroende av energi. Växter och vissa bakterier kan fånga och utnyttja solens energi för att bygga upp sina celler och dess delkomponenter som fetter, sockerarter och proteiner. Den energi som på så sätt blir bunden i dessa molekyler kan användas av djur och människor genom att vi konsumerar dem. För att energin skall kunna utnyttjas av kroppen måste den frigöras och konverteras till en för cellerna användbar energikälla och denna omvandling sker till största delen i mitokondrierna. Mitokondrier finns i alla kroppens celler undantaget några få speciella typer, exempelvis röda blodkroppar. Det har föreslagits att det ursprungligen var bakterier som för många miljoner år sedan började leva i ett samspel med celler, som inte kunde producera sin egen energi, och att bakterierna därefter utvecklats till de mitokondrier som nu finns i cellerna i kroppen. Stora sockermolekyler som stärkelse i till exempel potatis bryts ned till mindre molekyler som upptas av tarmen och transporteras till cellerna. Här fortsätter nedbrytningen med den så kallade glykolysen genom att sockermolekylen glukos delas och bryts ned till pyruvat. Pyruvat transporteras till mitokondrien där den ingår i den så kallade citronsyracykeln där ytterligare nedbrytning sker. Under denna process frigörs elektroner och protoner som överförs till två bärarmolekyler. De kan sedan avge elektronerna till andningskedjan som är en serie proteiner som transporterar elektronerna mellan sig för att till slut avge dem till en syrgasatom som då omvandlas till vatten. När elektronerna vandrar mellan proteinkomplexen frigörs energi som används till att pumpa ut protoner eller vätejoner till mellanrummet mellan mitokondriens två membran. På så sätt byggs en gradient upp mellan det inre av mitokondrien med låg koncentration av protoner och membran-mellanrummet med hög koncentration. Då alla atomer strävar efter att vara jämnt fördelade uppstår en kraft som utnyttjas av det femte komplexet i andningskedjan. Här kan protonerna komma tillbaka till insidan av mitokondrien och när de passerar genom detta komplex används kraften till att sammanfoga en ADP-molekyl med en fosfat-molekyl för att bilda ATP. Denna kan sedan transporteras runt i cellen för att användas som kroppens "batteri" vid energikrävande processer. Om det uppstår fel i mitokondrien kan det leda till energibrist i cellen. Detta påverkar i sin tur cellens olika funktioner som inte kan upprätthållas och som yttersta konsekvens kan energibristen leda till celledöd.

Sepsis brukar i vardagligt tal kallas för blodförgiftning. När en bakterie bryter igenom kroppens normala försvar startar en infektion. Kroppen börjar bekämpa denna infektion med hjälp av immunförsvaret samtidigt som den försöker hindra den från att sprida sig vidare. Om bakterierna är för många eller om kroppens immunförsvaret fungerar dåligt kan dock infektionen tränga igenom försvarsmekanismerna och sprida sig till hela kroppen. Vid sepsis är det egentligen inte bakterierna som sådana som



orsakar den mesta skadan (även om de orsakar viss skada) utan det är det kraftfulla immunförsvaret som bär det största ansvaret. När infektionen blivit generell och övergått i sepsis blir de flesta av kroppens organ påverkade med en försämrad funktion som resultat. Patienter blir förvirrade och får sänkt medvetande för att hjärnan är påverkad, får lågt blodtryck pga. att hjärtat pumpar sämre och att kärlen inte kan hålla samman, urinproduktionen går ned pga. njurpåverkan etc. Om infektionen behandlas och patienten får det stöd som behövs kan kroppen återhämta sig och organen återfå sin funktion. Orsaken till att organen slutar fungera är inte klarlagd men en hypotes är att mitokondrierna blir påverkade vid sepsis i en sådan grad att det uppstår energibrist i cellen som då inte kan utföra sina normala funktioner vilket får till konsekvens att organen slutar fungera. Energibristen är dock inte så grav att cellen eller organen dör då de efter hand bevisligen kan återhämta sig igen.

I de studier som ingår i den här avhandlingen har vi studerat mitokondriefunktionen hos patienter med sepsis och hos friska kontroller.

Ett problem när man skall studera mitokondrier vid sepsis är att man helst av allt skulle vilja undersöka dem i de organ som är mest påverkade vilket oftast skulle betyda hjärna, hjärta, lungor och njurar. På grund av de risker som finns med att ta ut provmaterial s.k. biopsier från dessa organ är detta inte möjligt och man får då istället använda andra vävnader. Tidigare studier har mest använt sig av muskelbiopsier. Vi har istället fokuserat på de mitokondrieinnehållande celler som cirkulerar i blodet dvs. trombocyter och vita blodkroppar.

I den första studien satte vi upp en metod för att analysera mitokondriefunktionen i trombocyter och undersökte hur den normala funktionen ser ut i olika åldergrupper, skillnader mellan kön samt hur länge vi kunde spara blodet utan att resultaten försämrades. Vi undersökte dels blod från navelsträng hos nyfödda, dels vanliga blodprover från barn samt från vuxna i både Sverige och Japan. I våra resultat kunde vi se att analysmetoden var mycket stabil mellan de olika grupperna och att vi kunde spara blodet i ca 24 timmar utan att det påverkade resultaten påtagligt.

I den andra studien tog vi blodprover från patienter med sepsis och jämförde resultaten från friska frivilliga personer. Blodproverna togs tre gånger under den första veckan som patienterna var inlagda på intensivvårdsavdelningen (IVA). Vi undersökte trombocyterna i patienternas egen plasma och i en vanlig buffertlösning och såg då att mitokondrierna som analyserades i plasman mädde sämre då de hade ett ökat läckage av protoner i sitt innermembran. Samtidigt såg vi att mitokondriernas funktion ökade successivt under veckan som gick så att de vid sista provtillfället fungerade nästan dubbelt så bra jämfört med kontrollerna. Ett annat fynd var att de patienter vars mitokondriefunktion ökade mest hade en högre dödlighet tre månader efter att de hade varit inlagda på IVA.

I den tredje studien gick vi vidare från trombocyter till vita blodkroppar. Dessa celler har en central roll i immunförsvaret och är aktiverade för att bekämpa infektionen i

sepsis. I flera studier har man konstaterat att längre fram i det septiska förloppet klarar de inte av att upprätthålla sin normala funktion och man har även här misstankar om att mitokondrierna kan vara involverade. Vi utgick från samma protokoll och tog prover från nya patienter med sepsis och isolerade fram de vita blodkropparna från blodet och undersökte mitokondriefunktionen. Liksom hos trombocyter fann vi att funktionen ökade under veckan som gick och tolkade det som tecken på stimulering av mitokondriell biogenes (ökad funktion eller mängd mitokondrier). Resultaten styrktes också genom att vi mätte tre olika mått på mitokondriemängd som alla ökade under analysperioden. Till skillnad från trombocyterna hittade vi inte något ökat läckage för protoner över innermembranet i vita blodkroppar från septiska patienter.

Att mitokondriers mängd och/eller funktion ökar kallas biogenes. Flera faktorer som produceras och frigörs vid sepsis kan potentiellt hämma mitokondriefunktionen samt även inducera mitokondriell biogenes. I den sista studien mätte vi nivåerna av ett utvalt antal av dessa signalämnen s.k. cytokiner samt indirekt även nivån av en gas, kväveoxid (NO) i plasman från de septiska patienterna. Vår hypotes var att nivåerna i blodet skulle korrelera med den mitokondriella respirationen så att de som hade högst nivå av cytokiner eller NO också var de som ökade mest i sin respiration. En av cytokinerna, IL-8 visade sig samvariera med mitokondriefunktionen i de prover som var tagna under de sista dagarna i den veckan vi undersökte patienterna. Det är således möjligt att IL-8 kan vara med i regleringen av mitokondriens ökade funktion i sepsis men fler studier är nödvändiga för att bekräfta detta.

Sammanfattningsvis så har vi sett att initialt i sepsisförloppet verkar det finnas en viss negativ påverkan på mitokondriefunktionen i trombocyter genom ett ökat läckage över innermembranet samtidigt som det sker en stimulering av mitokondrierna att öka sin funktion. Denna stimulering av mitokondriefunktionen fann vi även i vita blodkroppar där vi däremot inte kunde se några tecken till försämrad funktion vare sig tidigt eller sent i förloppet. Nivån av endast en av tio undersökta cytokiner i blodet samvarierade med mitokondriell funktion och även om flera av dessa ämnen har kapacitet att stimulera till ökad mitokondriefunktion är regleringen uppenbarligen mer komplex än ett direkt samband.

Trombocyter har visat sig fungera utmärkt för att bestämma mitokondriefunktion i sepsis och vi tror att tekniken kommer att visa sig användbar vid studier av ett antal andra sjukdomar och tillstånd.



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