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ON BIOENERGETIC FAILURE IN SEPTIC SHOCK: LACTATE KINETICS AND MITOCHONDRIAL RESPIRATION

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Lactate kinetics and mitochondrial respiration

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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My work consists of two parts: of the one which is here, and of everything which I have *not* written. And precisely this second part is the important one.

Ludwig Wittgenstein

To my family

ABSTRACT

Septic shock, a dysregulated immune response to an infection, is a major cause of mortality and morbidity in patients treated in intensive care units (ICUs). The cause of the organ dysfunction seen in sepsis is still not fully elucidated, but a disrupted intracellular metabolism, a bioenergetics failure, has been suggested as a possible mechanism. In this thesis we investigate two aspects of these metabolic alterations – mitochondrial dysfunction and lactate metabolism.

Compromised mitochondrial function and damaged mitochondria are described in septic ICU patients and various animal septic models. In our study we investigated if plasma from septic patients was able to alter the respiratory function of mitochondria of rat skeletal muscle. We examined this through incubation of isolated mitochondria as well as incubation of permeabilized muscle cells, but could not detect any difference in respiration compared to mitochondria incubated in control plasma.

Elevated concentrations, and impaired clearance, of plasma lactate are known to correlate with increased mortality in patients with septic shock. We examined the development of plasma lactate in septic shock patients treated in our ICU to determine the optimal rate of hourly reduction to use for prognostication. All patients treated for septic shock with plasma lactate >2mmol/L and treatment with vasopressors during 2015 and 2016 (n= 104, mortality 34%) were included. Lactate values from all blood gas analyzes, until normalization (<1.5 mmol/L) or 24 hours, whichever came first, were included. Changes in lactate concentrations between each sampling point were used to determine the mean hourly lactate reduction in percent. Mean reduction <2.5%/h and admission lactate ≥4mmol/L provided the best cut-off values for prognostication of poor outcome. Of the 22 patients with admission lactate >4mmol/L and decrease <2.5%/h, 14 died within 60 hours of ICU admission.

To study lactate metabolism, production as well as consumption, we examined two different approaches using intravenous administration of ^{13}C-labeled lactate, which can be distinguished from endogenous lactate through laboratory analysis. First we examined a continuous infusion of ^{13}C-lactate in healthy subjects. Samples were drawn from arterial and femoral venous lines and a skeletal muscle biopsy was taken at tracer steady state. An infusion of adrenaline was used to increase plasma lactate (from 1 to 4 mmol/L) and sampling was repeated after three hours. Skeletal muscle was a net contributor of lactate and adrenaline increased maximal mitochondrial respiration by 30%. The muscle biopsies showed a large variability and will not be used in clinical studies on septic patients.

We then investigated a less invasive protocol for studying whole body lactate metabolism. First healthy volunteers were administered a bolus dose of ^{13}C-lactate and a total of 43 blood samples were drawn during 2 hours. 10 ICU patients were then investigated using the same protocol. This protocol rendered similar values for lactate rate of appearance as other methods in healthy volunteers. ICU patients with normal lactate concentrations showed similar lactate metabolism as healthy volunteers. Simulations showed that blood samples can be decreased from 43 to 14 with the same accuracy.

We conclude that the protocol using infusion and arterio-venous sampling yield more information, but is more invasive and may be suitable for smaller physiological studies while the bolus approach is simpler and may be useful in larger cohorts. We plan on using these approaches to examine lactate kinetics in patients with septic shock. Other patient groups that may be interesting to study in the future includes post cardiac arrest, traumatic brain injury or postoperatively to major surgery.

LIST OF SCIENTIFIC PAPERS

I. **The effect of plasma from septic ICU patient on healthy rat muscle mitochondria**
Grip J, Jakobsson T, Tardif N, Rooyackers O
Intensive Care Medicine Experimental (2016) 4:20
DOI 10.1186/s40635-016-0093-2

II. **Lactate kinetics and mitochondrial respiration in skeletal muscle of healthy humans under influence of adrenaline**
Grip J, Jakobsson T, Hebert C, Klaude M, Sandström G, Wernerman J, Rooyackers O
Clinical Science (2015) 129, 375–384 doi: 10.1042/CS20140448

III. **Optimal cut-off for hourly lactate reduction in ICU treated patients with septic shock**
Promsin P, Grip J, Norberg Å, Wernerman J, Rooyackers O
Manuscript/submitted for publication

IV. **Lactate kinetics in ICU patients using a bolus of ^{13}C-labeled lactate**
Grip J, Falkenström T, Promsin P, Wernerman J, Norberg Å, Rooyackers O
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BPM	Beats per minute
Co-A	Coenzyme A
CS	Citrate synthase
DCA	Dichloroacetate
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
ED	Emergency department
ER	Endoplasmic reticulum
E_t	Enrichment at time = t
DO_2	Oxygen delivery
HR	Heart rate
ICU	Intensive care unit
IVIG	Intravenous immunoglobulins
J	Youden's index
LDH	Lactate dehydrogenase
LPS	Endotoxin (lipopolysacharide)
MAP	Mean arteriall pressure
MCT	Monocarboxylate transporter
MPC	Mitochondrial pyruvate carrier
MRT	Mean recidence time
mtDNA	Mitochondrial DNA
PBIC	Peripheral blood immune cells
PBMC	Peripheral blood mononuclear cells
pCO_2	Partial pressure of carbon dioxide
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
Pa	Pascal
pO2	Partial presssure of oxygen

Q	Pool size of substrate
R_a	Rate of appearance
RCR	Respiratory control ratio
RCT	Randomized clinical trial
R_d	Rate of disappearance
RhAPC	Recombinant human activated protein C
ROS	Reactive oxygen species
SA	Specific activity
SAPS 3	Simplified acute physiology score 3
$ScvO_2$	Central venous saturation of oxygen
SIR	Svenska intensivvårdsregistret
SSC	Surviving Sepsis Campaign
SGP	Strain gauge plethysmography
SOFA	Sequential organ function assessment
TNF-α	Tumor necrosis factor alpha
TEM	Transmission electron microscope
VO_2	Oxygen consumption
w.w.	Wet weight

1 INTRODUCTION

Intensive care units (ICU:s) care for the most critically ill patients of the hospital. The care is largely focused on optimizing the body's ability to heal, and maintain homeostasis during recovery. Patients treated in the ICU may have different diagnoses and underlying diseases, but the intensive care aspects are more often than not very similar, including metabolic alterations and need for hemodynamic and respiratory support to ensure adequate oxygenation throughout the body. All of these aspects are crucial in caring for patients with septic shock, the leading cause of mortality in the ICU.

1.1 DEFINITIONS OF SEPSIS

By its very nature sepsis is difficult to fully understand. It is a syndrome in which an inadequate immunological host response to an infection is leading to (multiple) organ failure. Sepsis has been defined through international consensus meetings. In 1992 the "Bone criteria" (1) were established and these remained mostly unchanged at the 2001 International Sepsis Definitions Conference (2). These definitions are based on clinical presentation where the response to an infection causes the host to fulfill at least two out of four systemic inflammatory response syndrome (SIRS) criteria (Table 1). If hypotension is present it is defined as severe sepsis and if blood pressure is not normalized by fluid resuscitation the patient has septic shock.

Table 1. SIRS criteria as used in definitions of sepsis

Systematic Inflammatory Response Syndrome (SIRS) Criteria	
Body temperature	$< 36^0$ C or $> 38^0$ C
Heart rate	> 90 beats per minute
Tachypnea	Respiratory rate >20 breaths / min or $pCO_2 < 4.3$ kPa
White blood cell count	$> 12 \times 10^3$ or $< 4 \times 10^3$ /mm^3 or presence of >10% immature neutrophils

pCO_2 partial pressure of CO_2 in arterial blood

As sepsis is associated with high mortality, finding patients in risk of deterioration to pool hospital resources are crucial, but costly. It is therefore necessary to avoid over- or under-diagnosis to allocate these resources to those in most need. Problematically, almost 50% of in-hospital patients fulfill two or more SIRS criteria at least once during their hospital stay (3) while 12% of patients treated for severe sepsis in the ICU do not (!)(4). To reduce this hazardous mismatch, sepsis was redefined in in a new consensus document 2016, Sepsis-3 (5). Unlike previous efforts, this definition is based on patient presentation and outcome from data registers. Sepsis was retrospectively specified and parameters predictive of outcome were identified. The SIRS criteria as well as the term "severe sepsis" are no longer used and sepsis is instead defined as "life-threatening organ dysfunction caused by dysregulated response to infection" (5). Organ failure is defined as an increase in Sequential Organ

Function Assessment (SOFA) score of two or more from baseline values. SOFA is a scoring system where the disruption in six different organ systems are scored, and has previously been used for prognostication of ICU patients (6, 7) (Table 2). According to Sepsis-3, patients are in septic shock when requiring vasopressors to maintain mean arterial pressure (MAP) >65 mmHg and have a plasma lactate >2 mmol/L, after adequate fluid resuscitation. The broad term "adequate" is however not more exactly specified.

At present time both of these definitions are in use and all septic patients within this thesis are defined with the 2001 conference consensus.

Table 2. Sequential Organ Failure Assessment (SOFA) score

Parameter	0	1	2	3	4
GCS	15	13-14	10-12	6-9	<6
P_aO_2 / F_iO_2 kPa	≥ 53.5	< 53.3	< 40	< 26.7*	< 13.3*
Blood pressure **	MAP ≥ 70mmHg	MAP < 70mmHg	Dopamine <5 or Dobutamine	Dopamine 5-15 or Adrenaline ≤0.1 or Noradrenaline ≤0.1	Dopamine >15 or Adrenaline >0.1 or Noradrenaline >0.1
Platelets, x 10^3/µL	≥ 150	< 150	< 100	< 50	< 20
Bilirubin, µmol/L	< 20	20 – 32	33 – 101	102 – 204	>204
Creatinine µmol/L	<110	110 – 170	171 – 299	300 – 440	> 440
or Urinary output				< 500ml / 24hrs	< 200 ml / 24 hrs

GCS Glasgow Coma Scale P_aO_2 Partial pressure of oxygen in arterial blood F_iO_2 Fraction of inspired oxygen * with respiratory support ** All dosing of vasopressors in µg / kg / min

1.2 EPIDEMIOLOGY OF AN OVER- AND UNDER -DIAGNOSED CONDITION

Sepsis may present as a primary infection at an emergency department (ED), but it may also be secondary to surgery, bowel perforation, immunosuppression, indwelling catheters etc. Septic patients are therefore cared for by physicians in a variety of departments. This makes it difficult to correctly appraise the prevalence within a single hospital. Comparing hospitals and entire countries are is even more difficult as the inclination to report varies with awareness of the disease and other factors, such as imbursements for treatment, heterogeneity in definitions and quality of registers. It is, however, commonly accepted that severe sepsis and septic shock are the leading causes of death in the ICU and one of the major causes of in-hospital mortality in the western world. In the US, sepsis is approximated to cause 265 000 deaths annually (8), but the estimations of incidence varies between 300-1 031/100 000 inhabitants/year depending on methods for data analysis (9).

Germany sees an increase in yearly sepsis in-hospital incidence between 2007 and 2013 from 256 to 335/100 000 inhabitants. Of these, a larger portion were classified as severe sepsis (27% vs 41%) and mortality from this condition decreased by 2.7% to 24.3% causing almost 68 000 deaths in 2013 (10). Similar trends are reported from Australia and New Zeeland between 2000 and 2012. During the study period the fraction of ICU admissions caused by severe sepsis/septic shock increased from 7% to 11%. Mortality decreased by approximately

1.3% annually and went from 35% in 2000 to 18% in 2012 (11). *Is this all due to improved identification and treatment?*

Unfortunately it is not that easy to say. Data from the Swedish Intensive Care Registry (SIR) (12) from 2008-2013 shows a decrease in both 30-day (32% to 29%) and 90-day mortality (35% to 32%). The decrease is notably less than in Australia and New Zeeland, even though the countries all have access to advanced health care and the registers have a coverage >90%. The difference may be attributed to different admission criteria to the ICU, which may also have changed during the study period in Australia and New Zeeland as the percentage of patients with sepsis increased. In Finland, with a health care system more similar to Sweden, both the incidence of severe sepsis and septic shock, and the in-hospital mortality (28%) are more similar to the Swedish data (13).

Levy *et al* compare outcomes between Europe and the US, as a follow-up on the Surviving Sepsis Campaign (SSC). The surprisingly large difference in mortality (41% vs 28%), disappears when patient data are adjusted for severity of disease, indicating different study populations on the two continents (14) and further highlights the difficulties in comparing data. The global burden of sepsis is almost impossible to estimate, as data from low- and middle-income countries are scarce, and extrapolated data from high-income countries should be interpreted with great caution. A meta-analysis of 27 studies from seven high-income countries found a yearly incidence of 437 and 270 per 100.000 for sepsis and severe sepsis, respectively. Extrapolation of these data would estimate 5.3 million deaths yearly worldwide (15).

Even though it is almost impossible to compare absolute numbers in large databases, and historical data is scarce and hard to translate, most experts agree that mortality in severe sepsis and septic shock is decreasing due to increased awareness, earlier recognition and standardization of treatment.

1.3 TREATMENT OF SEPSIS

Sepsis is a major cause of morbidity and mortality in ICU:s. During the last two decades more focus has been put on early identification and initiation of resuscitative treatment. Rivers *et al* published a milestone paper in 2001, showing a decrease in mortality from 45% for patients receiving standard care in the ED to 30% in the patients randomized to 6 hours early goal directed therapy (16). This, rather aggressive, resuscitation protocol has since then been challenged, but there is no doubt of its impact on early identification and treatment of septic patients worldwide. More recently, three multicenter randomized controlled trials (RCT:s) compared different protocols for resuscitative sepsis treatment and found no difference between them, indicating that the key to improved survival is early identification and standardization of care, rather than which of the existing protocols you are using (17-19).

Much of the improvements in the treatment of sepsis in the last decade can be attributed to educational efforts and international consensus of the Surviving Sepsis Campaign (SSC). SSC published their first guidelines in 2004 (20). This first edition was heavily influenced by the recent success of the Rivers trial protocol. The guidelines have been updated every 4th year, with the last full version being published in 2016 (21). The guidelines are comprehensive but are summarized in protocols, consisting of bundles with items that should be completed within three and six hours of disease presentation. In the latest bundle update this has been shortened to one hour (Table 3) (22). Implementation of, and adherence to, these bundles has been shown to decrease mortality in observational studies in high- (23-25) and middle-income countries (26). However, concerns have been raised that the guidelines are adapted to primarily western countries' standard of care and disease panorama, as many of the interventions are unavailable in low-income countries (27), and not enough emphasis are put on comorbidities such as HIV and tuberculosis (28).

Table 3. Surviving sepsis campaign 2018 1-hour bundle

Measure lactate level. Remeasure if initial lactate level >2mmol/L
Obtain blood cultures prior to administration of antibiotics
Administer broad-spectrum antibiotics
Begin **rapid administration of 30mL/kg crystalloid** for hypotension or lactate level ≥ 4 mmol/L.
Apply vasopressors if patient is hypotensive during or after fluid resuscitation to maintain MAP ≥ 65 mm Hg

Since the first guidelines, recommendations have remained strong for early recognition, blood cultures, timely and adequate fluid resuscitation and initiation of antibiotics (21). The parameters to be used to guide the resuscitation has however changed, as plasma lactate decrease is a better target than the previously used $ScvO_2$ (29, 30). Addition of lactate to the SSC-bundle has been shown to decrease mortality in sepsis (31). Since 2012, hydroxyl starch has been abandoned for fluid treatment (32, 33) and there is now greater caution concerning blood transfusions, since a transfusion trigger for hemoglobin (Hb) of 70g/L is non-inferior to 90g/L (34, 35).

The treatment of septic patients is constantly modified and re-evaluated as new knowledge becomes evident, both of the clinical manifestations and underlying pathophysiology. Currently there are ongoing discussions concerning the role of cortisone (36, 37), optimal amount of fluids (38) and optimal lactate clearance targets (39).

1.4 PATHOPHYSIOLOGY OF SEPSIS

The pathophysiology of septic shock is extremely complex and far from fully understood. Clinically, severe sepsis and septic shock are characterized by vasodilation, endothelial leakage and organ dysfunction, but the underlying cause is not yet established. Traditionally,

the hosts' immune-system has been considered the villain, with the pathogen playing the role of instigator, but this is probably an oversimplification (40). The systemic inflammatory response is a fine-tuned balance between pro- and anti-inflammatory pathways. It is suggested that the septic patient may initially react with a hyper-inflammatory response potentially followed by a hypo-inflammatory state or immunosuppression (40), at which point an infection might be even more dangerous than during the initial hyper-activation (41). The dangers of hypo-reactive sepsis might explain the observation that mortality in sepsis has a negative correlation with initial body temperature in the ED (42).

Even though the septic patient's immune system might be harmful, attempts to modulate or reduce the immune response have so far been unsuccessful in decreasing mortality. Intravenous immunoglobulins (IVIG) show no effect or even harm in high quality trials in mixed sepsis (43, 44), even though IVIG containing IgM may be efficient, especially in gram negative sepsis (45). Blocking tumor necrosis factor alpha (TNF-α), a pro-inflammatory acute-phase cytokine, increase mortality in clinical studies (46). The clinically mostly used immune modulator, recombinant human activated protein C (RhAPC, trademark Xigris), showed some promise in populations with high predicted mortality but was removed from market in 2011, after showing potential harm without benefit in larger populations (47).

The heterogeneity of both patients (with respect to genetics, past medical history and ongoing medication) and pathogens (site of infection, virulence of strain etc.) makes universal mechanisms hard to detect. Various animal models have been developed to investigate this, but these have often failed to be representative for the sepsis seen in ICU patients. In contrast to the clinical situation, these models most often consist of a homogenous populations of animals (young, healthy, in-bred) and septic shock induced with a single strain of bacteria or endotoxin (LPS) in dosages far above what is usually present in human infections, to ensure development of shock (and often death) in all animals. Animals have often been studied in the very early phases of sepsis (within hours) and been left untreated, whereas septic patients have received appropriate antibiotics, fluid resuscitation and intensive care. These differences are probably part of the explanation why several potential treatments, that have shown promise in animal studies, have failed to do so in human sepsis (48, 49).

Even though sepsis has been studied and characterized on molecular, cellular, intracellular and organ levels, the cause of organ failure in sepsis is not fully understood. Several pathways of the immune system are activated, and this is associated with a large stress response, hormonal disturbances, bioenergetic alterations, and vascular incompetence. Since several organs are failing simultaneously, a common mechanism has been suggested, such as oxygen depletion or simultaneous activation of apoptosis (programmed cell death). However, the degree of organ failure does not seem to be matched by histological findings describing larger degree of visceral damage (50). It has therefore been suggested that organ failure might be caused by a dysregulation of intracellular functions, such as the cells internal energy

turnover. Low ATP:ADP ratios and mitochondrial alterations (51) as well as an increased production of reactive oxygen species (ROS) (52) and decreased levels of antioxidants in plasma (53, 54) are seen in patients with septic shock. Metabolic alterations on the whole body level are also described, with increased plasma concentrations of amino acids, glucose and lactate, the latter showing a strong correlation to adverse outcome. In summary this bioenergetic dysregulation, or failure, is suggested as a potential cause of organ failure during sepsis, which we try to examine closer within this doctoral project.

1.5 MITOCHONDRIAL FUNCTION IN HEALTH

The mitochondrion is a key organelle in all eukaryotic organisms. Its bacteriological ancestry is still evident in that it carries its own DNA (mtDNA), although this only makes up for 13 of the approximately 1500 proteins present in human mitochondria (55). Even though the mitochondria play a role in several intracellular functions, such as apoptosis and intracellular calcium metabolism, they are best known as “the power house of the cell”, as they generate most of the cell’s ATP through oxidative phosphorylation, and for formation of ROS. ROS are used for intracellular signaling, but may also cause harm in form of oxidative damage (56).

Due to the continuous demand for energy substrates, the oxidative phosphorylation and mitochondrial biomass are tightly regulated by the intracellular milieu, such as NAD^+:NADH and AMP:ATP ratios or acetyl-CoA levels (55, 56), as well as hormones such as glucocorticoids and insulin (57).

Structurally, mitochondria are made up of two phospholipid membranes, a porin containing smooth outer membrane and a folded, less permeable, inner one (Figure 1). The intramembranous space between them has an electrochemical composition similar to the intracellular cytosol, while the matrix (space enclosed within the inner membrane) has a higher concentration of enzymes and an electrochemical gradient. In the matrix, pyruvate enters the Krebs cycle (citric acid or tricarboxylic acid cycle) as it fuses with Coenzyme A (CoA) to form acetyl-CoA at the pyruvate dehydrogenase complex (PDC). In Krebs cycle, NAD^+ and FAD are reduced to NADH and $FADH_2$, respectively. These high-energy substrates are then oxidized in complexes I-IV of the electron transport chain, located in the inner membrane, while pumping H^+ molecules into the intramembranous space. This electrochemical potential is then utilized in complex V (ATP-synthase), phosphorylating ADP into ATP, as H^+ are flowing back in to matrix.

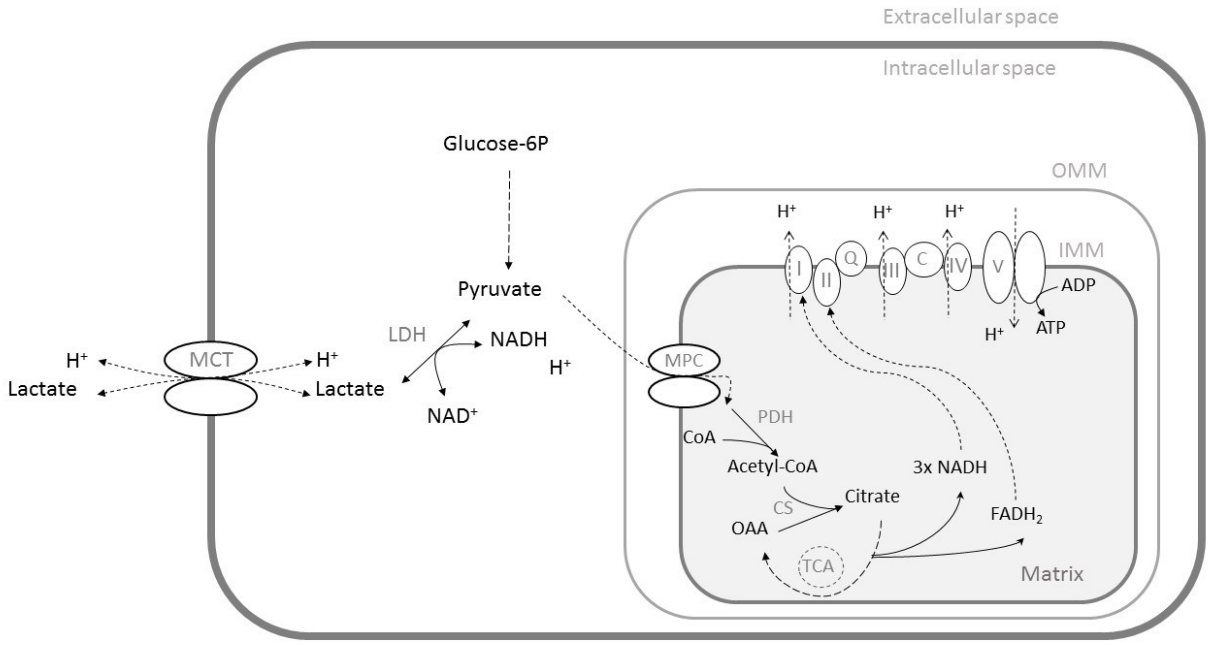

Figure 1. Cell containing mitochondria, schematic representation of key metabolic concepts referred to within this thesis. *ADP* Adenosine diphosphate, *ATP* Adenosine triphosphate, *C* Cytochrome C *CoA* Coenzyme A, *CS* Citrate Synthase, *FADH* Flavin adenine dinucleotide, *IMM* Inner mitochondrial membrane, *LDH* Lactate dehydrogenase *MCT* Monocarboxylate transporter, *MPC* mitochondrial pyruvate carrier *NADH* Nicotinamide adenine dinucleotide, *OAA* Oxaloacetic acid *OMM* Outer mitochondrial membrane, *PDH* Pyruvate dehydrogenase *Q* Coenzyme Q, *TCA* Tricarboxylic acid (Krebs) cycle.

Glycolysis, where one molecule of glucose is broken down into two molecules of pyruvate, takes place in the cytosol of cells. Pyruvate can then either be converted into lactate, by lactate dehydrogenase (LDH) in the cytosol, or transported into the mitochondrial matrix through the mitochondrial pyruvate carrier (MPC) proteins (58), and then enter the Krebs cycle. The Krebs cycle can also be fueled by fatty acids. Long-chained fatty acids react with CoA and are then transported into the mitochondrial matrix. There they are subjected to β-oxidation, where the long chains are shortened into two carbon molecules which reacts with CoA and enters the cycle as acetyl-CoA, similarly to pyruvate.

1.6 MITOCHONDRIAL DYSFUNCTION IN SEPSIS

The fact that multiple organs are failing in septic shock has led to the suggestion that there might be a common mechanism involved. Findings in septic human and various animal models has led to the suggestion that mitochondrial dysfunction might be a possible cause of organ failure.

1.6.1 Mitochondria in clinical and human experimental sepsis

There are great difficulties in studying mitochondrial function in septic, hemodynamically unstable, ICU patients. Patients are presenting at different time points during the day as well as during the cause of their disease, which makes comparisons difficult to fully interpret. Logistically there are challenges in designing study protocols that do not interfere with ongoing care but also ethical considerations in obtaining consent from critically ill (often unconscious) patients, whom often have an increased risk of bleeding. Sampling of intracellular organelles from visceral organs are extremely difficult, and therefore there are minimal amount of data from these organs. Most data are instead from more easily accessed organs, such as muscle or blood cells, which may not necessarily represent what is happening in the solid organs. For more information about the measurements presented below, see the method section.

ATP concentration are lower in skeletal muscle of non-surviving septic ICU patients compared to survivors. This is accompanied with decreased complex I activity, and tendency to ($p=0.05$) increased complex IV activity in the non-surviving group (unchanged complex II and III activity, all activities expressed as ratio per CS activity) (51). In contrast, citrate synthase (CS) activity decrease 30-40% in both leg and respiratory muscle of patients with septic shock throughout their ICU stay, but with unchanged complex activity, rather suggesting fewer rather than dysfunctioning mitochondria (59, 60). At the same time, the mitochondrial gene expression and protein synthesis rate remain unchanged, suggesting that mitochondrial damage and protein degradation must be increased, rather than a decrease in mitochondrial genesis (61). In fact, early activation of mitochondrial biogenesis in muscle seems to be associated with improved survival in septic patients (62). When healthy volunteers are given a bolus injection of endotoxin (LPS), CS and complex I activities are increased in skeletal muscle biopsies compared to biopsies taken before the LPS challenge, indicating an initial up regulation (63).

The state 4 respiration of intact platelet mitochondria increase in septic ICU patients throughout the first week from ICU admission (day 6-7 compared to day 1-2). The same changes are seen in permeabilized platelets when suspended in their own plasma as well as in preservation solution. Both maximal respiration and respiratory control ratio (RCR) are higher in platelets from survivors than non-survivors (64). Peripheral blood immune cells (PBIC) of septic patients gradually increase their respiration as well as mitochondrial content throughout the first week after ICU admission compared to healthy controls (65). Peripheral blood mononuclear cells (PBMC) have higher *ex vivo* oxygen consumption as compared to controls, interestingly the oxygen consumption of the healthy control cells increase after incubation with plasma from septic patients (66). However, permeabilized PBMC:s of septic patients from day 1-2 of ICU admission have lower state 3 respiration compared to healthy volunteers in a severity of illness dependent manner (67). PBMC:s from septic patients show a lower oxygen consumption and complex activity compared to healthy controls (68). When isolated mitochondria from skeletal muscle of healthy humans are incubated with plasma

from septic patients there is a tendency, although not significant, towards decreased mitochondrial respiration (68). A more recent finding is that mitochondrial response to sepsis vary in different populations of immune cells. Monocytes have a lower CS activity, compared to healthy controls, but higher complex I, IV and ATPase activity expressed per unit of CS. B-cells, on the other hand, have a higher complex I, unchanged ATPase, and lower complex IV activity while T-cells had higher complex I and ATPase activity and unchanged complex IV activity compared to controls (69).

The mitochondrial analysis in immune cells should be interpreted with some caution, especially when healthy volunteers are used as controls, as these samples probably represents different populations of cells. In experimental studies, when healthy controls are exposed to LPS, immune cells decrease in the central circulation within the first hours of exposure, to then increase in later phases. The different subclasses of immune cells also show different temporal patterns (70). These results indicate migration of the initial PBMC:s and a subsequent increase in "fresh" immune cells, which are not necessarily comparable samples. In that sense the study from Belikova *et al* is interesting as it indicates that something present in plasma from septic patients seem to affect blood cells from healthy controls to express changes similar to that of septic patients (66).

In summary, the data support that mitochondria in human sepsis in skeletal muscle and blood cells are affected, but the mechanisms regulating these processes are not fully established. Immune cells seem in general to have an up regulated mitochondrial activity/oxygen consumption, which seems reasonable as they should have a greater metabolic demand when being activated to react to a possibly lethal infection.

The effect seen in skeletal muscle mitochondrial are interesting but seems to differ from the circulating immune cells, with mostly a decrease in mitochondrial metabolism, except maybe during the initial phase. Mitochondria from other sources therefore give little indication about what happens in the solid visceral organs known to fail in sepsis, such as heart, liver and kidneys. Rapid postmortem biopsies of kidney and heart from deceased septic patients (n=44) have been compared to trauma and cancer patients (kidney) and transplant and brain-dead patients (heart). Heart biopsies do not show a large degree of cellular damage or mitochondrial dysmorphology, assessed by electron microscopy. Kidneys from septic subjects, however, have a high degree of localized cellular damage (in 78% of cases) accompanied by hydropic mitochondria and autophagosomes, although most tubular cells remain undamaged. These changes are not seen in the control groups, but the authors conclude that the degree of cell death does not correlate to the degree of organ dysfunction (50).

There are of course limitations to post-mortem studies, and the internal organs of living septic patients are, as mentioned, difficult to study. However, they are more readably available in experimental animal models.

1.6.2 Mitochondria in animal experimental sepsis

During the last decades, huge efforts have been made to investigate mitochondrial (dys-) function in various septic animal models. A wide range of species and septic insults have been used. The obvious advantages of using animal models are that sampling can be standardized, homogeneity between subjects increase the chances of finding general mechanisms, availability of sampling from solid organs and logistical considerations (important, since swift and accurate handling are vital for the mitochondria to survive until examination). Mitochondria are, evolutionary speaking, old structures with great similarities between species, but signaling and potential immune system mediators may differ between various animals and humans.

Even though several studies show that mitochondria are affected, the heterogeneity of species, insults, methods being used (see method section), organs investigated, and timing of sampling makes these difficult to compare (71). Respirational function decrease in long term septic models (often >16hrs) (72) in several different organs, but some, mainly short term models, actually describe an increase in respirational function of e.g. hepatic mitochondria (73).

The conflicting evidence is illustrated by the fact that Mervyn Singer establish that associations between degree of mitochondrial dysfunction and outcomes in sepsis are “well-recognized” but causality not have been proven (74). While on the other hand, Jeger *et al* summarize mitochondrial function in sepsis as “variable, organ specific and changing over the cause of the disease”, concluding that the current data from studies in animals does not support mitochondrial function as the main denominator in multiple organ failure of septic humans (71). Even though both papers are based on largely the same data, the latter is designed as a systematic review, methodologically showing the inconsistencies in findings throughout organs and species. Most studies analyze a vast number of organs and parameters and when pooled together a majority of analyzes actually show no effect. The effects that have been shown are often smaller than the variability of the methods used (71).

A dormant, hibernating (temporary down regulated), state of mitochondria in sepsis has also been suggested, but in a review of literature we could not find solid support for this claim (75).

The limitations of the animal models used up until today has been addressed in the section on pathophysiology above. Altogether, the results from decades of animal studies have not led to any strong evidence on what is actually happening in mitochondria of solid organs in human sepsis. Hopefully this problem will be addressed in future studies as the consensus initiative on pre-clinical animal studies recently published new guidelines, which aims to standardize sepsis studies in animals (76-79).

1.7 LACTATE METABOLISM IN HEALTH

Lactate ($C_3H_5O_3^-$) is a three-carbon substrate formed by reduction of pyruvate ($C_3H_3O_3^-$) + H^+ and simultaneous oxidation of NADH to NAD^+, by LDH. LDH is a family of enzymes, found mainly in the cytosol of cells, each containing four subunits of either M (muscle) or H (heart) type. LDH-1 is found in the heart and contains four H-subunits, while LDH-5 only contains M-subunits and is found in striated muscle as well as in liver. The other variations (LDH-2 to LDH-4) exist in various tissues around the body (80). At any given moment the direction of the reaction is determined by the intracellular ratio of pyruvate/lactate and NADH/NAD^+, but the different isoforms of the enzyme have different affinity for these reactions. LDH-1 (found in heart, but also in smaller concentration in slow twitch muscle fibers) favors conversion of lactate to pyruvate, while LDH-5 (predominant form in muscle and liver) favors formation of lactate from pyruvate.

Therefore, in normal physiological conditions, skeletal muscle acts as a net contributor of lactate at rest as well as during initial exercise (81). Depending on the type of muscle fiber and exercise, muscle show different lactate release, uptake and utilization patterns during prolonged exercise (82). The heart is, on the other hand, a net consumer of lactate both at rest and during exercise (83). In septic rats the availability of lactate is essential for heart function and survival (84). The brain can, depending on conditions, be either a net consumer or contributor of lactate (85, 86).

Although most organs (those containing mitochondria) can oxidize lactate for ATP production, only the liver and kidneys are able to convert lactate back to glucose through the process of gluconeogenesis. This mainly takes place during starvation/post absorptive state and is stimulated by the insulin/glucagon relationship as well as substrate availability (87).

Lactate was first described more than 200 years ago, but its metabolism has been more thoroughly investigated in the last decades as it is no longer viewed as strictly an anaerobic product but as a metabolic intermediary with a complex metabolism. Lactate is co-transported in and out of cells together with H^+ through the actions of monocarboxylate transporter (MCT) proteins. Both MCT1 and 4 are isoforms found in skeletal muscle, while the MCT1 dominates in the heart. MCT1 has a higher affinity for lactate (K_m approximately 5 mM) than MCT4 (K_m approximately 20mM), indicating that MCT1 has a greater role in transport of lactate into cells, while MCT4 predominantly exports lactate from the cell (88). A high prevalence of MCT1 could be seen as more useful for cells utilizing lactate as an energy substrate through oxidation, whereas a high concentration of MCT4 would be more useful in cells who contribute lactate to the circulation (and thereby serves as energy substrate in other organs) (89). MCT4 is present in all types of skeletal muscle, but MCT1 is found predominantly in slow twitch type 1 fibers (90). MCT4 in the only MCT expressed in white blood cells, known to rely heavily on glycolytic metabolism, and are net producers of lactate (91). Hypoxia is also known to induce MCT4 expression (92).

1.8 LACTATE METABOLISM IN SEPSIS

The association between increased plasma lactate and poor prognosis in severe sepsis/septic shock has been known and studied for a long time (93-95). An increased baseline value is a strong prognosticator for increased in-hospital mortality. Plasma lactate above 4 mmol/L is often used as a cut-off, but septic patients with more subtle plasma lactate levels of 1.4-4 mmol/L also show increased mortality (96-100). A lower cut-off of 2.5 mmol/L has been suggested (101), and in the new sepsis criteria, plasma lactate >2 mmol/L, after resuscitation, is used in the definition of septic shock (5).

At higher concentrations, plasma lactate has an even stronger correlation to poor prognosis. Septic patients, not taking metformin, with plasma lactate >10mmol/L have a mortality of a staggering 88%, compared to 57% mortality for those on metformin medication (102).

Compared to the elevated concentrations at baseline, changes in plasma lactate over time show an even stronger predictive correlation, as a swift decrease or normalization (often measured at 6h after start of resuscitation) is associated with markedly lower mortality (39, 103-106). Even a slight decrease in plasma lactate within two hours from start of resuscitation is correlated with halved mortality compared to an increase from baseline (15% vs 36%) (107). Sustained high lactate levels in septic shock strongly indicates a critical condition, plasma lactate >4 mmol/L after 24h of intensive care is associated with a mortality of 83.3% (108).

Initially, the presence of lactate was most often attributed to tissue hypoxia (109), probably because its classical role as end product in anaerobic glycolysis, but also that fluid resuscitation, which increases oxygen delivery (DO_2), decreases lactate concentration (110). During the 90´s, this notion was challenged as increased DO_2 does not lead to increased oxygen consumption (VO_2) while plasma lactate concentrations decrease (111). Further support is given by the fact that Dichloroacetate (DCA) decrease plasma lactate by stimulating pyruvate dehydrogenase activity, and thereby increasing oxidation of pyruvate, in septic patients (112). A more nuanced picture of lactate as a complex but vital intermediate in a patient's metabolic response to stress induced by septic shock started to emerge, backed by experimental evidence in animal models as well as healthy humans and septic patients (113).

Pyruvate dehydrogenase (PDH) is the enzyme responsible for the conjunction of pyruvate and CoA into Acetyl-CoA, the main precursor in the tricarboxylic acid circle (TCA). In skeletal muscle of septic rats this enzyme is inactivated (phosphorylated) in significantly greater extent compared to both sterile inflammation and control animals after 3 and 7 days, simultaneous to development of hyperlactatemia (114).

Injection of 2ng/kg of endotoxin to healthy humans increase plasma adrenaline levels compared to placebo (0.7 ±0.1 vs 0.3 ±0.1 nmol/L) simultaneously as lactate increases (2.5 ±0.5 vs 0.9 ±0.1mmol/L) (115) and plasma potassium decreases (3.3 ±0.1 vs. 3.8 ±0.1mmol/L). These changes mimic those when rats are injected with E. coli (116), where

intracellular Na^+/K^+ ratio also decreases. Further support for the role of Na^+/K^+ase is that septic patients export lactate from skeletal muscle to plasma, as assessed through micro dialysis, but this export halts with local administration of Oubain (a known inhibitor of Na^+/K^+ase) (117).

Na^+/K^+ase activity in skeletal muscle is known to be stimulated through adrenergic β-receptors and local β-blockade decreases lactate export into plasma (118).

Levy *et al* show that plasma lactate in endotoxemic rats can be decreased by either selective β_2-antagonism (ICI-118551) or DCA. When treated with both these substances simultaneously, plasma lactate diminish totally and the animals die within 80 minutes. However, when substituting the lack of lactate production with an infusion of exogenous lactate, the animals survive in a similar manner to those with ordinary endotoxemia (118).

The role of catecholeamines in lactate release has also been shown in humans as lactate is higher when epinephrine is used instead of dobutamine-noradrenaline in vasopressor-dependent septic shock (without affecting cardiac index, blood pressure or DO_2) (119).

All together this not only shows that lactate is not a strict anaerobic product but also that it might play an important role as an energy substrate during metabolic stress. In fact it is even suggested as a possible therapeutic substrate (120). Failure to utilize lactate might be more detrimental than an initial high level in a critical condition, as lactate decrease is a better prognosticator than initial value. When septic ICU patients without elevated plasma lactate are infused with sodium lactate (1mmol/kg) to increase plasma concentrations (approximately 1.5 mmol/L), survivors return plasma lactate to baseline values faster than non-survivors (121). Similarly septic patients with slight hyperlactatemia clear this challenge slower than normolactemic patients, showing similar rates of lactate production but decreased rates of clearance from plasma (122).

There has previously been one attempt to describe lactate kinetics in septic patients with ^{13}C-labeled lactate (123). In this study healthy volunteers (n=7), patients with septic shock (n=7) and cardiogenic shock (n=7) are compared using an ambitious protocol with a 6 hour infusion of ^2H-labeled glucose, indirect calorimetry, and an increasing infusion of ^{13}C-labeled lactate. Initially 10μmol x kg^{-1} x min^{-1} of labeled lactate was infused during the third and fourth hour of the protocol and then 20μmol x kg^{-1} x min^{-1} for the remaining two hours. In this study, lactate rate of appearance (R_a) is 11.2 ±2.7 μmol x kg^{-1} x min^{-1} for healthy volunteers, 26.2 ±10.5 μmol x kg^{-1} x min^{-1} for septic and 26.6 ±5.1 μmol x kg^{-1} x min^{-1} for cardiogenic shock patients during the lower infusion rate. The R_a:s for the higher infusion rate are not presented as this infusion rate is likely to affect the lactate kinetics. However this might be the case also for the lower infusion rate as the exogenous lactate administered equaled half of the endogenous production rate and increased the plasma lactate itself (see method section below).

Even though the association between increased plasma levels of lactate, and poor lactate "clearance", and mortality in septic shock are firmly established, the actual metabolism of lactate in septic shock is still poorly studied. This may in part be due to the difficulties in performing experimental physiological studies in critically ill patients, but also partly due to lack of good, feasible methodological protocols. Within this doctoral project we have tried to investigate possible study protocols to describe lactate kinetics in septic patients.

2 AIMS

In our studies we aimed to

- Investigate if a factor in plasma from septic ICU patients can alter mitochondrial respiration *ex vivo*

- Investigate the usefulness of a 3-compartment model to describe muscle lactate kinetics in humans using ^{13}C-labeled lactate

- Investigate the effect of an adrenaline challenge on lactate metabolism at the whole body level as well as in skeletal muscle in healthy volunteers

- Examine mitochondrial respiration in response to exogenous adrenaline in healthy volunteers

- Investigate lactate kinetics of healthy volunteers and mixed ICU patients using a ^{13}C-labeled lactate bolus technique

- Investigate the optimal cut-off rate for short term lactate decrease in ICU patients treated for septic shock

3 METHODS

3.1 STUDY SUBJECTS

3.1.1 Study I

In the first part (Ia) ICU patients (n=10, 8 alive at 30 days) with severe sepsis or septic shock were recruited within 24 hour of ICU admission. Controls, matched for sex and age, were recruited to be sampled in the postoperative unit after undergoing elective surgery. In the second part (Ib) a new population of ICU patients with severe sepsis/septic shock were recruited (n=10, 8 alive at 30 days) in a similar manner. A new group of controls, healthy volunteers matched for sex and age, were recruited and given a small financial compensation for participation.

All participants were recruited after oral and written informed consent (or by next of kin when appropriate in the ICU population). Participation meant consenting to donation of one blood sample (10 ml) and permission for the researchers to gather clinical information from electronic charting systems. This information included past and present illness, ongoing care and outcome (mortality) after 30 days.

3.1.2 Study II

A total of 12 healthy male volunteers were included in the pilot (n=4, mean age 24 years) and main study (n=8, mean age 33 years). All subjects were screened with questionnaires, health examinations and routine blood samples. One subject was excluded from participation due to an increased risk of bleeding (spontaneous PK(INR) of 1.4) and referred to primary health care for further investigation. Study subjects were given a financial compensation for participation (3500 SEK, approximately €350).

3.1.3 Study III

Data on all adult (≥18 years) patients treated in the ICU of Karolinska University Hospital, Huddinge during 2015 and 2016 for severe sepsis/septic shock was extracted from the electronic charting system. Those with other infectious diagnosis were screened for fulfillment of sepsis criteria. Patients with initial lactate of >2mmol/L and noradrenaline treatment were included in the study (n=104). Patients only participated through retrospective collection of clinical data through electronic charting system and consent was waived by the regional ethics committee.

3.1.4 Study IV

In the first part, IVa, healthy volunteers (n=6, mean age 34, 2 female) were screened for past and present illness and oral and written consent were obtained. Healthy volunteers were given a small financial compensation for loss of income (500 SEK, €50). In study IVb ICU patients

(n=10, mean age 60, 1 female) were recruited for participation. Inclusion criteria were age ≥18 years, ability to consent to participation, planned ICU stay >24 hours, in-dwelling arterial catheter and no planned examinations (e.g. CT-scans) outside the ICU for the duration of the 2 hour protocol. ICU patients received no financial compensation.

3.2 ANIMAL HANDLING

In study I, in-bread rats were used, Sprague-Dawley (6-8 weeks old, 150-250g). Animals were cared for in our laboratory animal facility according to the Helsinki declaration for treatment of animals with enrichment and constant access to food and water. Initially penthobarbiturate was used for euthanasia, but due to inconsistencies in results, and indications in literature that penthobarbiturate affects mitochondrial respiration in rat liver (124), we moved away from this method. All data from experiments presented within this thesis comes from animals euthanized with CO_2. Daily maintenance and animal care were handled by trained animal facility staff and all experimental animal handling was performed by the same person for standardization (Jonathan Grip).

In our experiments only tissue (soleus muscle) were used for experiments. To reduce the risk of damaging the mitochondria it is of great importance to minimize time between euthanasia and storing the muscle specimen on ice in preservation buffer. From previous studies, in our and other groups, we used an upper time limit of 4 minutes. None of the specimens used for the studies within this thesis had a time between euthanasia and chilled storage of more than 3 minutes.

3.3 ETHICAL CONSIDERATIONS

Several ethical aspects have been considered within the scope of this thesis. In study I participants were not put through any medical risks, as participation only meant donating 10 ml of blood. The personal information obtained were also kept to a minimum and all data was kept in a coded manner. As to the animal handling, we tried to minimize suffering while still maintaining scientific quality (as poor scientific methodology would be even more unethical), e.g. we moved away from the recommended method of intraperitoneal injection to use CO_2 to decrease risk of mitochondrial damage. However, in our subjective perception, the animal seemed more stressed from the injection than gas euthanasia. Animals had free access to food and water, enrichment and company. As far as logistically possible we tried to minimize the time the last rat in the cage was alone by performing those experiments in the same day as the second to last rat.

In study II and IV healthy subjects were studied and exposed to both invasive procedures and given a hemodynamically active compound (II). This mandates careful ethical considerations as they are put through risks without any chance of benefit from participation. We therefore

took great care in the screening process (and one subject was excluded). All experiments were performed in close proximity to the ICU, all invasive procedures were performed by experiences staff, and a physician was present at all time. We also used a stepwise approach by performing a pilot study with careful titration of adrenaline to make sure to minimize risks and uncomfort for the subjects. Subjects were given financial compensation, however this also mandates some ethical considerations as there is a risk that subjects would expose themselves to risks they would not otherwise do if this compensation was too large.

Study III was a pure retrospective register study and the subjects faced no medical risks through participation. On the other hand sensitive data was handled without their consent or knowledge. Great care was taken in that none of the data was kept in any identifiable way and that only one code key was available and kept locked in a safety cabinet. Despite these efforts, and the fact that we think the knowledge gained far outweighs the breech of personal integrity, we cannot exclude that some of the subjects would have disagreed if asked.

3.4 LABORATORY METHODS

3.4.1 Mitochondrial content and enzyme activity

There are several different approaches to assess mitochondrial content and function, which may contribute to the heterogeneity in literature that is seen. Volume measurements through transmission electron microscope (TEM) is the gold standard to assess mitochondrial content (125). This is however a highly limited resource as it is both expensive and requires highly specialized staff. Therefore other methods are being used and TEM has been compared to mtDNA and cardiolipin content as well as complex IV and citrate synthase (CS) activity. Of these cardiolipin and CS activity correlate best to TEM (125), as the amount of mtDNA varies between mitochondria. CS is an enzyme present in the mitochondrial matrix and active in the TCA cycle. Maximum CS activity are considered stable in most situations, although acute changes has been described, e.g. in an endotoxin challenge (63) and after a short exercise bout (126).

Activity of each respiratory complex, and other mitochondrial enzymes, can be assessed by spectrophotometric based enzyme assays. The advantage with this approach is that only small amounts of tissue homogenates are needed, but there are limitations in that maximal enzymatic activities are measured, which may not necessarily reflect the activity in vivo (127). Often the activities are normalized for CS, to adjust for varying amount of mitochondria in the samples. Several studies on mitochondria in ICU patients are using these methods, either alone or as a complement to respiratory analysis (68, 69).

3.4.2 Mitochondrial respiration

Oxygen is reduced to water at complex IV as the last step in the electron transport chain before ADP is converted to ATP at complex V. Analyzing the rate of oxygen consumption during the conversion of ADP to ATP gives a good assessment of the function of the entire mitochondria and different complexes, depending on the presence of substrates or inhibitors.

State 1 respiration is the oxygen consumption in absence of any substrates, state 2 respiration is measured in the presence of substrates but absence of ADP. The respiration in presence of ADP is called state 3 and state 4 is the oxygen consumption after all ADP has been converted to ATP (Figure 2). A high state 2 or state 4 respiration is often seen as damaged or uncoupled mitochondria. State 3 respiration divided by state 4 respiration, respiratory control ratio (RCR), is often used as an indicator of the quality or damage of the mitochondria being examined (127-129).

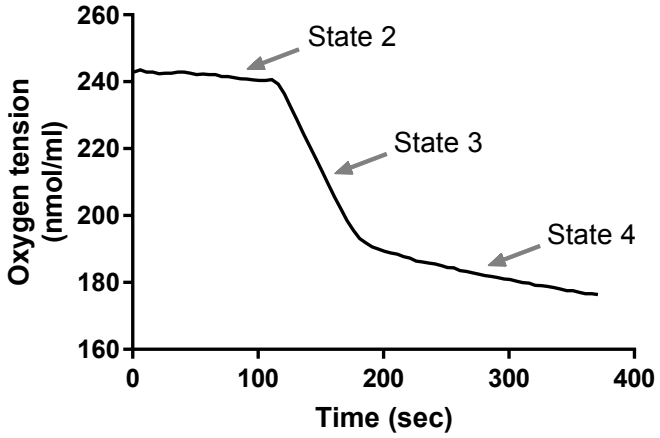

Figure 2. Schematic illustration (based on actual experiment) for mitochondrial oxygen consumption using an Oxygraph.

In our experiments we analyzed mitochondrial function using an Oxygraph (Hansatech DW1; Hansatech, King's Lynn; Norfolk, UK), a small chamber containing a Clarke-electrode, measuring oxygen tension in the solution. Constant temperature was maintained by a flow of heated water (25^0 C) circulated in the chamber wall. The main advantage of this method is that the entire respiratory function of still living mitochondria are analyzed. However, it is a sensitive method that requires rather large specimen (minimally 100 mg of tissue), only allowing for short examinations (as oxygen is then depleted) and is quite sensitive for temperature and sample preparation. This method allows for assessment for function of the individual complexes by specific inhibiting substances and/or substrates, but we did not use these within the thesis, but rather studied the intact respiration of the entire mitochondrial structure. Oxygraphs are also used in studies on PBMC in septic patients (66).

Another way to measure mitochondrial respiration is through high-resolution respirometry (129) which has been used in several studies on mitochondria in sepsis (64, 65). An advantage of this method is the possibility to measure ROS formation simultaneously (130).

3.4.3 Mitochondrial preparations

3.4.3.1 Mitochondrial isolation

In study I and II we analyzed isolated mitochondria from skeletal muscle of rats (I) and human subjects (II). The isolation processes were similar between the two studies which included disintegration of the muscle with scissors and homogenization with a protease to dissolve peptide bonds. After washing in a preservation buffer, a series of centrifugations followed and the isolated mitochondria were dispended in a preservation buffer. After incubation (in study I), the solution was put in the Oxygraph chamber and energy substrates were added. Respiration was measured and state 3 respiration started by addition of ADP (Figure 2). The process is described in more detail in each study respectively (including incubations in study I). The entire preparation process and measurements were time consuming and sensitive, as the mitochondria might be damaged by changes in temperature or mechanical strain. We were therefore limited to a maximum of two experiment per day (or a case and a control in study II). As each isolation contains a series of manual preparations we made sure to standardize these as much as possible and that the same person performed all experiments within each study (Jonathan Grip in study I and Tove Jakobsson in study II). Although the method is well described and have been used in studies in patients and healthy athletes, concerns have been raised that the mitochondria yielded with the isolation technique may represent a healthier or more normal subset (131, 132). This would probably mostly influence studies performed on mitochondria from subjects (or animals) in sick populations where the damaged mitochondria may have structural abnormalities. We do not think these potential limitations interfered in our first study as the mitochondria were isolated prior to the exposure, but we cannot exclude that this would influence the results in study II as those mitochondria were exposed to the stimulus prior to isolation. However we find it unlikely that the mitochondria would have been morphological disordered during this short exposure to adrenaline. We corrected all respirations from isolated mitochondria to units of CS activity to compensate for the various amount of mitochondria in each sample.

3.4.3.2 Muscle fiber permeabilization

As we did not find any effect of plasma from septic patients on the function of isolated mitochondria we performed a second series of experiments with incubation of permeabilized muscle fibers instead of isolated mitochondria. During the permeabilization process the lipid membranes of the cells are lysed with saponin allowing larger molecules to pass through but keeps large parts of the internal cell structure unaltered. An advantage of this over the isolated mitochondria, is that more of the intracellular milieu is preserved and the mitochondria can be studies in its natural position as an integrated part of cellular cascades and in close

cooperation with cytoskeleton, endoplasmic reticulum (ER) etc. Permeabilization also allows for longer incubation times as the mitochondria are stable through a 24 hour ice-cold storage (133). This would allow for a potential later effect to be discovered. Our protocol is described in more detailed in study I and is an adaptation of previously published detailed protocols (128, 134). We present the respiration as oxygen consumption per mg of wet weight in each specimen.

3.5 TRACER METHODOLOGY

The only way to quantify metabolic fluxes *in vivo* is through tracer methodology. In tracer studies an exogenous version (tracer) of the studied compound (tracee) is given, most often intravenously. The assumptions for using this methodology are that the tracer: 1) is metabolized the same way as the tracee 2) does not affect the metabolism of the tracee 3) can be distinguished from the tracee through analysis.

In order to fulfill the third criteria the tracer has to be labeled in some way. There are two main principles for labeling of tracers. The first is by using a radioactive compound and subsequently measure radio activity in the sample. Results are presented as specific activity (SA; Disintegrations per minute (DPM)/concentration). This method has the advantage that a smaller tracer dose can be used, but the downsides are potentially shorter shelf-life and the considerations (practical and ethical) of using radioactive compounds. The second alternative is by using tracers labeled with stable isotopes that can be differentiated from the tracee by measuring their mass through Mass Spectroscopy. This relationship is presented as enrichment (E):

$$Enrichment = \frac{tracer}{(tracee+tracer)} \qquad (1)$$

There are obvious advantages in not relying on radioactive isotopes, but the downside is that the more insensitive measuring techniques require higher doses of the tracer, potentially interfering with the assumption that metabolism is unaffected by the tracer itself. There is also naturally occurring "background" activity, which need to be compensated for through base line sampling. In our experiments we use stable ^{13}C-labeled lactate. ^{13}C is a naturally occurring isotope, constituting approximately 1.1% of all carbon. Incorporated in multi-carbon molecules in the body it is metabolized in the same way as "ordinary" ^{12}C. However when plants incorporate carbon from the atmosphere, ^{13}C diffuses slightly slower through membranes, at rates varying between different types of plants. This, and some enzymatic preferences, makes ^{13}C content higher in certain staple foods (as corn) than others (such as rice, wheat, soy beans and potatoes) (135). Depending on diet, subjects will therefore have different naturally occurring amount of ^{13}C-lactate, and baseline sampling prior to tracer administration is crucial. Although this has been shown to complicate exercise studies (136), in our studies this is unlikely to interfere with the results.

Most often the tracer and tracee are sampled from an artery or a vein. The rate of appearance (R_a) is the velocity of which the tracee is transported into the sampled compartment (e.g. µmol/kg/min) from other tissues and the rate of disappearance (R_d) is the velocity at which the tracee is removed from that compartment. At a constant plasma concentration, R_a and R_d are identical. Clearance is the amount of plasma that is effectively cleared from the substance in a given unit of time (e.g. L/min). There are two principal ways to administer the tracer, through an intravenous bolus dose or a continuous infusion. The following are adaptations from the second edition of *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis* (137).

3.5.1 Bolus injection technique

When administering tracer as a bolus injection, there will initially be a high tracer to tracee ratio (Figure 3). As tracer and tracee disappears at the same rate (through consumption or transportation) enrichment will decrease only as additional new tracee appears into the sampled compartment. The enrichment at time point, E_t, can be described as:

$$E_t = E_{(0)}e^{-kt} \qquad (2)$$

Where $E_{(0)}$ is the calculated enrichment at time=0 and k is the rate constant of elimination, which is derived from:

$$k = \frac{lnE_{t1} - lnE_{t2}}{t_2 - t_1} \qquad (3)$$

By estimating $E_{(0)}$ the entire pool size (Q) of tracee can be calculated:

$$Q = \frac{Dose\ tracer}{E_{(0)}} \qquad (4)$$

Rate of appearance R_a can then be calculated as

$$R_a = k \times Q \qquad (5)$$

But can also be calculated from the area under the enrichment curve

$$R_a = \frac{Dose\ tracer}{\int_0^\infty E(t)dt} \qquad (6)$$

Mean residence time (MRT) can be calculated as

$$MRT = \frac{Q}{R_a} = \frac{1}{k} \qquad (7)$$

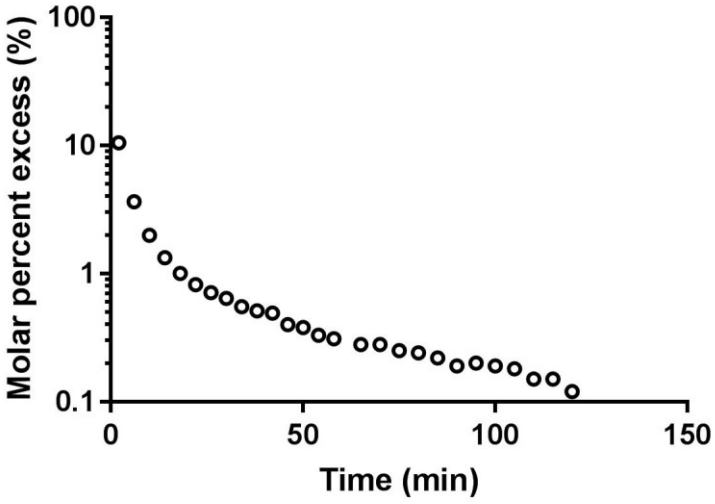

Figure 3. Example (adopted from experiment in study IVa) of decay in enrichment (molar percent excess) after bolus dose of ^{13}C-labeled lactate

3.5.2 Continuous infusion technique

In the case of continuous infusion of the tracer, the enrichment of plasma will gradually increase and eventually reach a steady state (Figure 4). The enrichment at a given time point (E_t) is given by the equation:

$$E_t = E_p\,(1 - e^{-kt}) \qquad (8)$$

Where E_p is the plateau enrichment. The rate constant (k) can be derived from sampling during the up slope of the curve, similar to the case with the bolus injection. By knowing the infusion rate of tracer (F), R_a is simply derived from measuring enrichment at the plateau phase:

$$R_a = \frac{F}{E_p} \qquad (9)$$

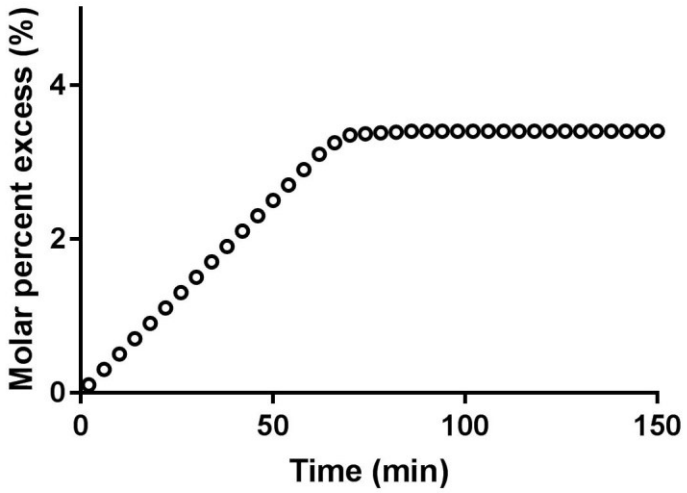

Figure 4. Example of enrichment (molar percent excess) as a function of time during a constant infusion of tracer.

Other kinetic parameters can thereafter be derived from the same calculations as above. There are several advantages to the infusion technique. Only a small amount of sampling is needed and it is well suitable to examine the results of an intervention, which may lead to a new steady state of enrichment. The obvious disadvantage is that it might take a long time before steady state of enrichment is reached, often several hours. To decrease this time an initial bolus dose of tracer, or a "prime", may be given at the start of the constant infusion. However, especially in diseased states or non-steady state situations, over or under-priming might cause a significant problem.

In the case of a constant plasma concentration, rate of disappearance (R_d) always equals R_a, and all the equations above are applicable. When plasma concentrations are changing throughout the experiment, equations for non-steady state (Steele equation) must be applied:

$$R_d = R_a - pV\ x\ \frac{C_2 - C_1}{t_2 - t_1} \quad (10)$$

Where pV is the distributional constant (for lactate 0.1 L/kg).

3.5.3 Non-compartmental modelling

In the examples above we have used the formulas as if the tracer and tracee are distributed in a single compartment. For most substances this is a simplification and the tracee are most often transported between more than two or more compartments, reaching equilibriums between these at different rates. If the tracer is introduced to the same compartment that is

being sampled non-compartmental modelling can be applied to account for *n* different equilibriums:

$$E_t = A\,e^{-akt} + Be^{-bkt} \ldots + Ne^{-nkt}$$

As noticeable in Figure 3, the decrease in enrichment occurs at different rates at different time points, faster directly after bolus injection and slower later in the experiment. The equation is therefore not best described with a first grade equation. With the help of computer software two, three or *n* exponential equations can be applied. These will "fit" the data with various accuracy and the simplest (least exponential) equation should be used.

3.5.4 Multiple compartments

By sampling from only arterial plasma the summation of the entire body's export and uptake of the tracee can be assessed (whole body kinetics). By sampling the concentration of tracee and enrichment at a venous site simultaneously to the arterial sampling, the uptake and release from the organ of the sampled vein can be assessed and (together with blood flow measurements) quantified. With addition of biopsies from tissue, the uptake, release and production in that tissue can be quantified (Figure 5). In study II we investigated the usefulness of such model for examining lactate fluxes over the leg (skeletal muscle) in humans, as previously described in anesthetized dogs (138).

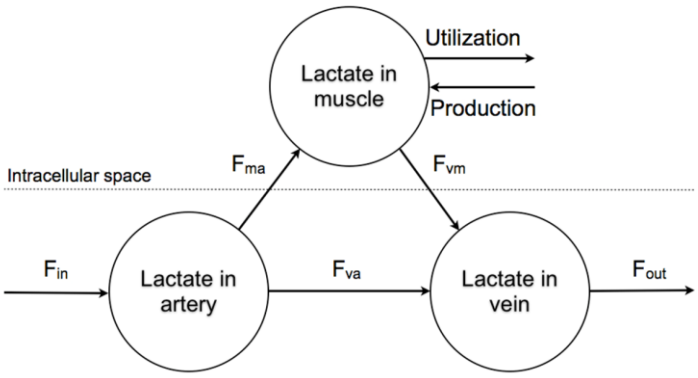

Figure 5. Schematic representation for lactate fluxes in skeletal muscle of the leg. *F* flux. Printed with permission from Clinical Science, Portland Press.

3.6 BLOOD FLOW MEASUREMENTS

To determine regional blood flow in the studied leg in study II, we used strain gauge plethysmography (SGP). In plethysmography a cuff is inflated to occlude the venous return in a limb and the rate of dilation of the distal limb can then be assessed through a water chamber

or (as in our case) by using an elastic strain-gauge, (139, 140). This is a method commonly used in physiological experiments in heathy (141) and critically ill subjects (142) with well described methodology (143). However, concerns have been raised that the pressure applied to occlude venous return, although lower than diastolic pressure, actually affect the arterial blood flow to the limb (144). We used a cuff pressure of 40 mmHg. As the diastolic pressure in the intervention part of our study was approximately 58 ±5 mmHg we find it unlikely that this had any significant effect. However, in hemodynamically unstable ICU patients, this may have a larger impact and alternative methods, such as Doppler ultra-sonography could be considered.

3.7 STATISTICAL METHODS

In study I all experiments were paired, as batches of mitochondria were the same for the sepsis and matched control plasma. In study II all subjects were examined before and after intervention (infusion of adrenaline) and data was therefore also paired. We could therefore apply paired statistical methods, such as Student's paired t-test.

In study III we analyzed retrospective data and compared survivors with non-survivors. For continuous data with normal distribution, student´s independent t-test was used, while Mann-Whitney U test was used when non-normal distribution was shown (by Shapiro-Wilks test). Categorical data was analyzed with Chi-square and Fisher´s exact test. Variables showing statistical significance in univariate analysis were included in a forward stepwise multivariate regression to exclude confounders. Receiver operating characteristics (ROC) curves were constructed and are under those area under ROC (AUROC) were calculated for 30-day mortality for baseline lactate concentrations and different values for hourly reduction of lactate. By calculation of the Youden´s index ((J) = sensitivity + specificity – 1) the optimal cut-off points for these values were derived.

In study IV we compared data from healthy volunteers and ICU patients. As these groups were not paired and equal variability could not be assumed, we used Welch´s two sided t-test for normal distributed data and Mann-Whitney U test for non-normal distributed data.

These groups may not be relevant to compare, but we are cautious by our interpretations of the p-values, and rather focus on the similarities between the groups. Pearson correlation between lactate concentrations and R_a were performed for the measurements in ICU patients.

3.8 STUDY PROTOCOLS

3.8.1 Study I

Ia) Healthy rats (6-8 weeks old) were sacrificed using CO_2. M. soleus were immediately harvested and put in a pre-chilled tube containing a preservation buffer. Mitochondria were isolated as described above and dissolved in an isolation buffer. The final homogenate containing mitochondria were divided into three parts. Two parts were incubated with either plasma from an ICU patient with severe sepsis/septic shock or an age and sex matched postoperative control for 30 minutes while kept on ice in a dark environment. Respiratory analysis of respiration was performed in an abundance of substrates at 25^0 C. The last part of the homogenate was immediately frozen and kept in -80^0 C until analysis of CS. Respiratory rates for each experiment were analyzed in a blinded fashion and corrected for CS activity in each sample.

1b) Healthy rats (6-8 weeks old) were sacrificed using CO_2. M. soleus were immediately harvested and put in a pre-chilled tube containing a preservation buffer. Muscle fibers were disintegrated with scissors and permeabilization of membranes was performed using saponin. Two portions of the sample were then incubated with plasma from an ICU patient with severe sepsis/septic shock for 30 and 120 minutes, respectively. Similarly, two portions were incubated with plasma from an age and sex matched healthy control before being analyzed for respiration. A fifth portion of the tissue sample was analyzed for respiration without any incubation to make sure that the mitochondria were not damaged during preparation of the muscle specimen. In those cases where the control sample showed signs of decreased mitochondrial function experiments were cancelled and redone another day. Respiratory rates for each experiment were analyzed in a blinded fashion and corrected for wet weight of the sample.

3.8.2 Study II

IIa) First we performed a pilot study to determine a safe and reliable protocol for increasing plasma lactate by a constant infusion of adrenaline. Four healthy male volunteers were screened for past and present illness. The fasting subjects received an arterial and a venous catheter. A primed continuous infusion of lactate was started together with a gradually increasing dose of adrenaline (0.02 $\mu g \times kg^{-1} \times min^{-1}$ up to 0.1 $\mu g \times kg^{-1} \times min^{-1}$). Blood samples were drawn every 15^{th} minute to follow lactate trends and for surveillance of pH and potassium levels.

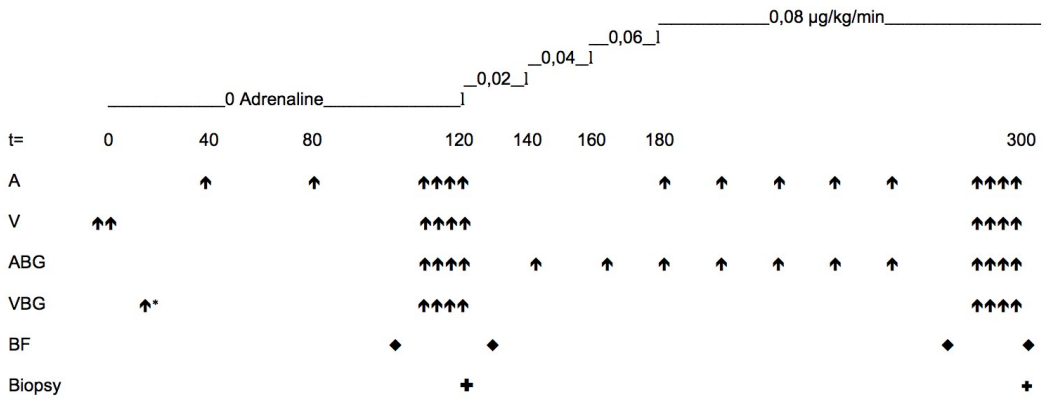

Figure 6. Finalized protocol for interventions and sampling for study IIb. Arrows indicate blood sample. *A* arterial blood sample, *V* venous blood sample, *ABG* arterial blood gas. *VBG* venous blood gas, *BF* blood flow measurement. Printed with permission from Clinical Science, Portland Press.

IIb) In the finalized protocol (Figure 6) eight healthy male volunteers were equipped with electrocardiographic and pulsoxiemtry monitoring, radial artery catheter (including non-invasive blood pressure monitoring), a femoral venous catheter and a peripheral venous catheter. Baseline samples were drawn and a primed continuous infusion of ^{13}C-labeled lactate (1 mg x kg^{-1} + 3.65 mg x h^{-1} x kg^{-1}) was started. After 3 hours, blood samples (arterial and venous) were drawn, a muscle biopsy harvested and blood flow measured twice. At each sampling, one sample was analyzed at a point of care blood gas analyzer and one sample centrifuged, snap frozen and kept in -80^{0}C. After this adrenaline was started (0.02 µg x kg^{-1} x kg^{-1}) and gradually increased during the first hour to a final rate of 0.08 µg x kg^{-1} x kg^{-1}. After 2 hours at this rate, the sampling was repeated and the adrenaline infusion stopped. Muscle specimens were immediately divided into two portions, one snap frozen in liquid nitrogen for future analysis of lactate and glycogen and one put in preservation buffer for mitochondrial isolation and analysis for respiratory function. After all subjects were included, plasma and muscle samples were defrosted and analyzed by GC-MS for determination of ratio between labeled and unlabeled lactate (see paper II for details on analyses).

3.8.3 Study III

After patients were screened for eligibility (see above), all arterial blood gas analyses drawn during patients ICU stay were extracted from the electronic charting system. All subjects blood gases were included until lactate normalization (<1.5 mmol/L) or until 24 hours from ICU admission, if lactate did not normalize during this period. As blood gases were drawn at various time points, changes in concentrations were divided by Δt to get an average lactate change per hour. Area under receiver operating characteristic curves were computed for baseline lactate and Δlac/h in relation to 30-day mortality. Optimal cut-off values were assessed by Youden's index (J). Forward stepwise multivariate logistic regression analysis was performed to investigate for confounding factors and factors predicting slow lactate decrease.

3.8.4 Study IV

IVa) Healthy volunteers (screened for past and present illness) came to the department after an overnight fast. An arterial and a venous catheter was inserted before baseline samples were drawn and a bolus injection of ^{13}C-labeled lactate (20 μmol x kg^{-1}) was administered over 20 seconds. Blood samples were drawn every 2 minutes during the first hour and every 5 minutes during the second hour. Samples were centrifuged and frozen and later analyzed for enrichment of ^{13}C-lactate. The same protocol was used in 10 ICU patients (IVb).

The decline in enrichment was assessed with an approach similar to those in previous works from our group, e.g. in the study of glutamine kinetics (145). The first points (2, 4 and 6 minutes) were extrapolated back to 0.5 minutes. From this point a line was drawn to the 0 coordinates to make up the boundaries of the "head" end of the curve. The tail end of the curve was transformed to the log domain and extrapolated to reach the baseline. These lines were then used to calculate area under the curve for enrichment, which was then used to calculate R_a (equation 6) and clearance for lactate. After this we examined the conformity between the protocol containing 43 samples and a minimally invasive one of 14 samples.

4 MAIN RESULTS AND DISCUSSION

4.1 STUDY I

In this study we examined the effect of plasma from septic patients on isolated rat muscle mitochondria (Ia) and permeabilized rat muscle fibers (Ib), as compared to postoperative and healthy controls. In neither of these experimental set-ups we could show a difference in effect between the septic and control conditions.

In Ia the RCR were good, 11 ±3.5 vs. 11 ±2.4, indicating good quality of the isolated mitochondria. The average maximal respiratory rates (state 3 respiration) were almost identical between the two groups (20.6 ±6.2 nmol O_2 x U CS^{-1} x min^{-1} for septic plasma vs. 20.7 ±8.4 for postoperative control group), Figure 7a.

In Ib RCR also indicated good quality of mitochondria (between 8.8 ±1.5 and 11.2 ±2.3 for the different groups). State 3 respirations were very similar between the groups, with the unincubated controls consuming 2.9 ±0.7 nmol O_2 x mg $w.w.^{-1}$ x min^{-1}, the septic patients 2.6 ±0.3 and 2.5 ±0.4 nmol O_2 x mg $w.w.^{-1}$ x min^{-1}, and the healthy controls 2.4 ±0.7 and 2.5 ±0.6 nmol O_2 x mg $w.w.^{-1}$ x min^{-1} for 30 and 120 minute incubation respectively, Figure 7b.

Initially we performed a series of pilot experiments on incubations of isolated mitochondria. There we observerd a tendency towards increased state 3 respiration and RCR after incubation with septic plasma compared to controls. However, these experiments had a large variability and somewhat high failure rate. After standardizations and some changes in the methodology (e.g. in methods of euthanasia) we had more stable success rate in obtaining high quality mitochondria and planned for a larger study. In the proposed study we aimed to elucidate which factor in plasma that could affect mitochondrial respiration. We recruited new ICU patients and decided to use sex and age matched post-surgical patients as controls, as these would be more similar to the patients than healthy volunteers. In this new study (Ia) we did not observe any differences in effect of the plasmas on the mitochondria.

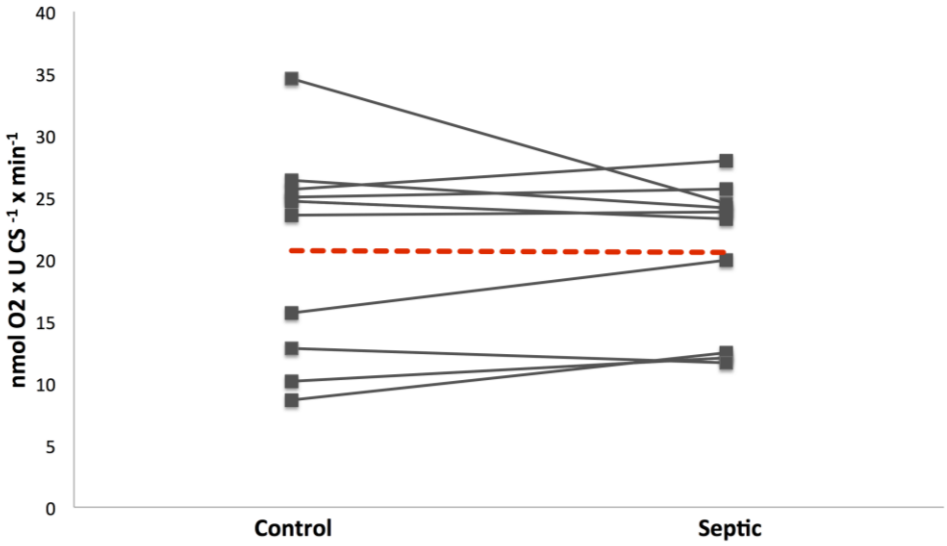

Figure 7a. State 3 respiration from healthy rat muscle mitochondria incubated with plasma from septic ICU and post-surgical patients.

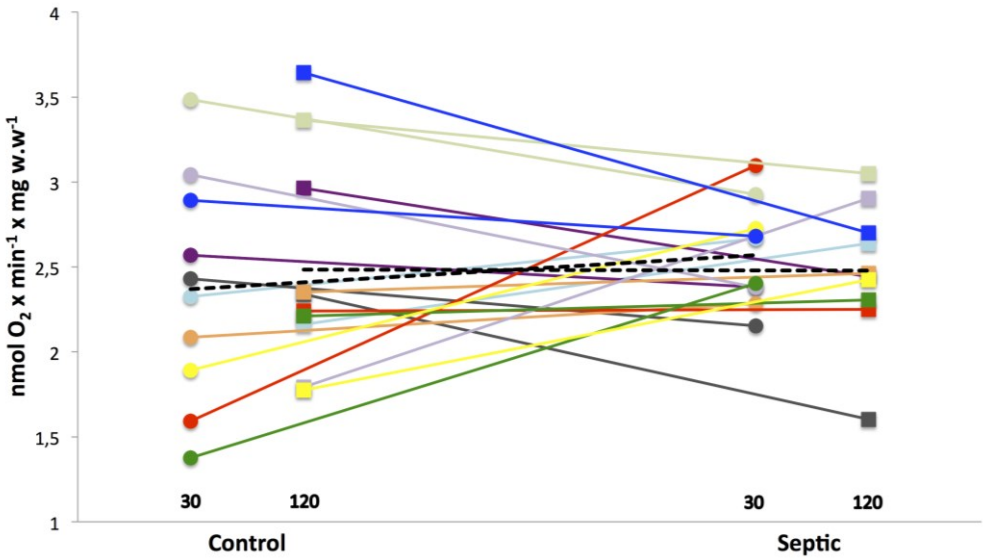

Figure 7b. State 3 respiration of permeabilized muscle fibers from healthy rat incubated with plasma from septic ICU patients or healthy controls for 30 and 120 minutes. Each color represents a paired patient and matched control. Dotted lines are means for each time point.

Pursuing a factor in these plasmas then seemed useless and instead we established the method with permeabilized muscle fibers to investigate if plasma could affect mitochondrial respiration when incubated in a more natural environment. This would also allow for longer incubation times. In this experiment we instead used healthy controls, to exclude any inflammatory mediators that could be present in both the postoperative and the septic patients.

Even though we examined our hypothesis that plasma from septic patients would affect mitochondrial respiration with two different approaches (isolated and permeabilized), with different controls and different durations, we found no tendency to an effect neither between groups nor between incubation times.

We believe this study was performed with good methodology rendering high quality mitochondria, and if there was an effect present, we would have discovered it. All examinations were blinded for the researcher determining the respiratory rates and performing the data analysis. A previously published study has seen a tendency, although not significant, to decreased oxygen consumption in isolated skeletal muscle mitochondria from healthy humans incubated with septic plasma (68), we could not replicate such tendency. This can however be caused by slight differences in methodology. Although both were incubating for 30 minutes, they used 37^0 C while we performed our incubations in a cold environment. We performed our experiments pair-wise, to compensate for the variation in the sensitive methods, which seems not to be the case in (68). When isolated swine liver mitochondria are exposed to LPS, the mitochondrial RCR decreases (146), but this effect could not be seen with the plasma from our septic patients. There may also be a difference between muscle and liver mitochondria as swine liver mitochondria show an increased state 4 respiration (and thereby decreased RCR) while the respiration of skeletal muscle mitochondria does not, during a prolonged LPS challenge (147).

There are of course limitations to this study, such as the use of rat tissue. Even though mitochondria are primitive structures, the rat mitochondria may not exactly replicate the response that would have been the case for human tissue. Another factor might be the time and temperature for incubation and analysis. We think that a warmer incubation environment would cause damage to the mitochondria and thereby introduce another confounding factor. We perform our analysis in 25^0 C, according to a standardized protocol (148) which has shown differences between groups in studies from other groups and ours (e.g. study II).

4.2 STUDY II

The main aim of this study was to investigate and validate a protocol to study skeletal muscle lactate kinetics in human subjects in a 3-compartment model, as suggested (149) and performed in anesthetized dogs (138). The results in the pilot study were used to design the final protocol.

The reasons for to studying lactate turnover over the leg was the strong indication that muscle is an important contributor to the increase in plasma lactate concentrations seen in septic shock patients (117). We wanted to make sure that the subjects had lactate concentrations similar to those seen in septic patients and chose to use adrenaline, which is known to increase plasma lactate concentrations but has previously not been studied in detail. Interestingly, catecholeamines seems to be involved in lactate release seen during sepsis as

well (150), but we do not consider this protocol a model for sepsis lactate metabolism, but merely a way of increasing lactate production to validate the method.

As expected during the adrenaline infusion, heart rate and plasma levels of adrenaline, glucose, lactate and venous saturation increased, while diastolic blood pressure, plasma potassium, calcium and pCO_2 decreased. Average plasma lactate increased from 1 to 4 mmol/L as expected, but with a larger range in the main study than in our pilot experiment.

Lactate kinetics were described for whole body, arteriovenous differences, as well as a 3-compartment model with uptake over the leg. Whole body R_a was 10.8 ±1.9 μmol x min^{-1} x kg^{-1} at rest, but tripled to 32.8 ±9.5 μmol x min^{-1} x kg^{-1} during the adrenaline infusion. When calculated for arterial-venous difference, lactate uptake in leg muscle increased during the adrenaline challenge, but not as much as lactate release, making muscle a net contributor to the lactate increase (0.37 ±0.44 μmol x min^{-1} x 100ml leg $volume^{-1}$). The muscle biopsies showed great variability, making the 3-compartment model difficult to fully interpret. Similarly, glycogen content analysis showed no difference and large variability, although adrenaline is suggested to stimulate glycogenolysis in muscle (151).

Mitochondrial state 3 respiration increased approximately 30%, adjusted for CS activity, after infusion of adrenaline. This seem to be novel findings in human but are similar to the effect of adrenaline in rat liver mitochondria (152). On the other hand, isolated swine mitochondria exposed to noradrenaline, dopamine or dobutamine show decreased respiration (146). These findings were somewhat surprising to us. Interestingly oxygen uptake and CO_2 release of the leg did not change during the adrenaline challenge, as would be expected from an increased mitochondrial respiration. This might be because the isolated mitochondria are examined in an environment with abundance of ADP, while the resting muscle has no consumption of ATP and therefore no extra ADP to fuel the oxidative phosphorylation. This can be thought of as the mitochondria being put in a state of readiness by the adrenaline, prepared to increase ATP production when intracellular energy is needed. This finding will have to be examined further for validation and clarification.

This was an advanced study from a logistical perspective, which demanded coordination between several persons drawing samples from several sites, handling these and performing measurements simultaneously. From this perspective we were satisfied with the results. We think the method was good in describing lactate metabolism for whole body kinetics as it rendered R_a at rest similarly to previously published data using continuous infusions of labeled lactate, 10.8 ±1.9 μmol/kg/min, compared to the healthy rested controls in clinical trials e.g. 9.6 ±2.2 (153) or 11.2 ±2.7 (123), but lower than published data in active healthy volunteers 17 ±2 (154) and elite skiers 18 ±3 (82). The arteriovenous difference showed that the leg (skeletal muscle) became a net contributor of lactate, which was expected. The estimated contribution of all skeletal muscles was however only 23% of the entire body's R_a at both rest and during adrenaline infusion (assuming 68g of muscle/100ml of leg tissue (155) and muscle constituting 40% of body weight). If we assume that the leg is a good

representation of all skeletal muscles in the body, this means that during adrenaline infusion in humans, other tissues also increase their lactate R_a. Still, since the muscle, in relative terms decreased the utilization of lactate (R_d), muscle becomes an increased net producer to the larger lactate pool during the adrenaline infusion.

The intracellular measurements, for lactate as well as glycogen concentrations, were associated with great variability making these results difficult to interpret. We did not expect this while designing the study and the biopsies did not show any macroscopic differences between them (which could have been indicating sampling from different compartments). Lactate enrichments in the muscle samples were low and we made several efforts, with cleaning muscle biopsies and measuring muscle values multiple times, to minimize the analytical variation, and we believe that this high variation is more the results of variation in the physiology, probably with local differences within the muscle. This could perhaps have been accounted for by taking multiple biopsies, but this would have increased the invasiveness of the study protocol.

The main aim of this study was to investigate the usefulness of a 3-compartment approach in ICU patients with septic shock. After publication we moved forward to construct such protocol. Based on the results yielded from the present study we concluded that the 3-compartment approach did not yield enough extra information, as compared to the arteriovenous sampling, to motivate the increased invasiveness of the muscle biopsies. We also introduced Doppler ultrasound for blood flow measurements, moving away from the SGP, as this method is less and less frequently used in clinical studies. We performed a small trial examining the correlation between the two methods in ICU patients. This showed good correlation for detection of changes in blood flow in stable ICU patients but significantly less so in hypotensive patients, receiving larger doses of noradrenaline (unpublished data).

4.3 STUDY III

As we aimed to study lactate metabolism in the septic patients treated in our ICU we first wanted to examine this population clinically in more detail. It is long known that lactate concentrations and clearances are associated with poor outcome, but we wanted to examine this in our patient material for a number of reasons. Most data sets reporting on lactate thresholds are from older data sets, using the previous definition of sepsis, while we wanted to have a cohort which fulfilled both the old and new criteria for septic shock. We also thought that our patients did not necessarily represent those previously studied, as we are a (1) Scandinavian (2) tertiary university hospital with few ICU beds in relation to hospital and community size and (3) have a large part of patients undergoing upper GI (including liver) surgery, or being treated for liver or haematological diseases.

In studying these patients, we thought it would be interesting to investigate if we could find an optimal cut-off for the hourly rate of which lactate decrease could be used for

prognostication as well as investigating what admission lactate was optimal to use in this setting. This could guide us to better define time periods and cohorts to be studied for changes in lactate clearance in relation to outcome in future studies of patients with septic shock.

A total of 1,188 lactate values from 104 patients were included in the analysis. Median baseline lactate value was 3.8 mmol/L (2.6 – 6.2) and median hourly reduction rate was 2.6% (-0.3 – 5.4).

The optimal cut-off points for predicting 30-days mortality for baseline lactate at ICU admission was 4 mmol/L and a rate of decrease was 2.5%/h, justified by the maximal value of the Youden's index (Table 4). Discrimination for 30-day mortality with lactate decrease and baseline lactate were similar (AUROC 0.694 (0.58 – 0.81) vs. 0.669 (0.55 – 0.79)).

Table 4. Youden´s index (J) for baseline lactate and hourly lactate reduction.

Cut-off values	AUROC (95% CI)	Sensitivity	Specificity	Youden's index
Baseline lactate > 3 mmol/L	0.56 (0.44 – 0.68)	0.74	0.38	0.12
Baseline lactate > 4 mmol/L	0.65 (0.53 – 0.76)	0.63	0.67	**0.30**
Baseline lactate > 5 mmol/L	0.65 (0.53 – 0.76)	0.51	0.78	0.29
ΔLact/h < 2 %/h	0.67 (0.56 – 0.78)	0.63	0.71	0.34
ΔLact/h < 2.5 %/h	0.68 (0.57 – 0.79)	0.71	0.65	**0.36**
ΔLact/h < 3 %/h	0.65 (0.54 – 0.76)	0.77	0.52	0.29

AUROC area under the receiver operating characteristic, *CI* confidence interval, *ΔLact/h* mean hourly reduction rate of lactate

Patients with baseline lactate >4 mmol/L and a slower decrease than 2.5%/h had a mortality of 70% within three days of ICU admission (statistically significant different compared to those with either lower baseline or greater decrease, $p<0.001$) as presented in Figure 8.

Lactate decrease < 2.5%/h, baseline lactate > 4 mmol/L, old age, and high simplified acute physiology score 3 (SAPS 3) were independent predictors of 30-day mortality

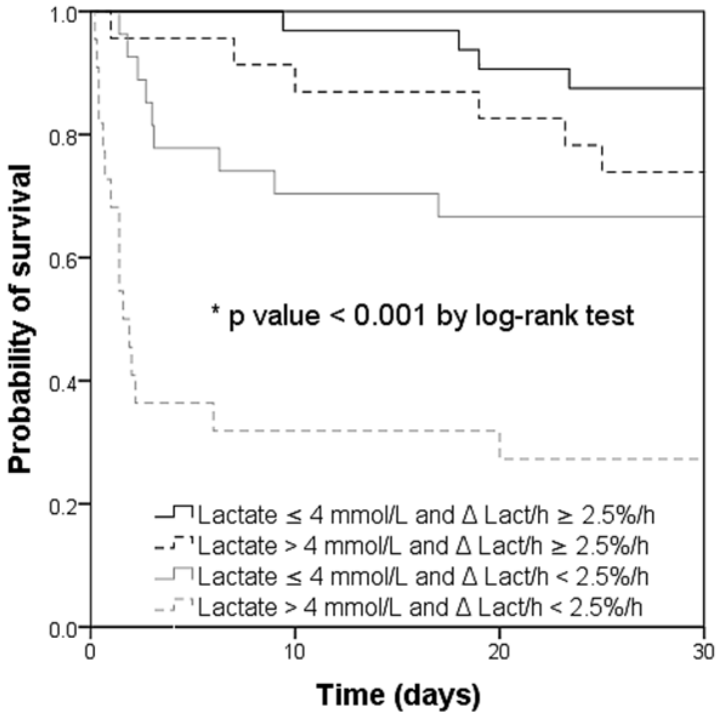

Figure 8. Kaplan-Meier survival curves comparing survival probability during 30 days following ICU admission 1) Lactate ≤ 4 mmol/L and ΔLact/h ≥ 2.5%/h (n = 32), 2) Lactate > 4 mmol/L and ΔLact/h ≥ 2.5%/h (n = 23), 3) Lactate ≤ 4 mmol/L and ΔLact/h < 2.5%/h (n = 27) and 4) Lactate > 4 mmol/L and ΔLact/h < 2.5%/h (n = 22). *ΔLact/h*: hourly reduction rate of lactate

Although it is well established that failure to clear an initial high plasma lactate concentration, our study contributes with a new perspectives as all patients fulfill both the old and new sepsis-3 criteria. We found it interesting that the "traditional" baseline value of 4 mmol/L is useful also in this setting as most patients are already resuscitated upon arrival to the ICU (as compared to baseline values in the emergency department). In fact, most of our patients arrive from other wards and thereby represent a different cohort then presented in most other studies. We also elucidated an optimal cut-off clearance point by a novel approach which was interesting but needs to be confirmed in future studies.

4.4 STUDY IV

After completing study II, we realized that this protocol would be useful for performing physiological studies in smaller cohorts, but difficult to use in larger studies. As it is dependent on simultaneous sampling, insertion of a femoral venous catheter and blood flow measurements it requires large staff resources and is investigator dependent. This would therefore most likely only be applicable during office hours, and considering the rate at which these patients appear at our unit (approximately 50 per year according to study III), recruitment of 10 patients would most likely take at least a year, considering vacations and holidays. We therefore wanted to examine a "smaller" protocol that could be used in larger populations of patients, preferably without invasive procedures and with a minimally need for

invasive blood sampling. This protocol should be able to be performed by a single person and without affecting ongoing intensive care.

We therefore performed a study to elucidate a minimally invasive protocol for describing whole body lactate metabolism in ICU patients, according to the method section above. Plasma lactate for healthy volunteers and ICU patients were 0.7 (0.4 – 0.9) vs 1.4 (0.6 – 4.9) mmol/L (median (range)), p=0.032 and R_a 12.8 ±3.9 and 22.7 ±11.2 µmol x min^{-1} x kg^{-1}, p=0.026. Lactate clearance was 1.57 ± 0.39 vs 1.10 ± 0.43 L/min, p=0.047, after correction for the lactate cleared through dialysis in the patient on continuous renal replacement therapy (CRRT). Approximately 10% the total AUC for enrichment over time were extrapolated (outside of sampling time) for ICU patients, showing the importance to include this is analysis. The ICU patients showed good correlation between plasma lactate concentration and R_a (r^2=0.85, p<0.001) and clearance (r^2=0.5, p=0.023). When ICU patients were dichotomized into groups with normal (n=6) and elevated lactate (n=4), the ICU patients with normal lactate concentrations had lactate kinetics very similar to healthy volunteers. Analysis showed that sampling could be decreased from 43 to 14 samples (100 to 30 ml) with preserved accuracy.

Our protocol gave results for lactate R_a for healthy volunteers similar to previously published data (se discussion from study II) using other methods as well as during the control conditions in study II, Figure 9. This supports the validity of using this approach to study whole body lactate kinetics.

All together, we think this is a feasible, reliable, and relatively non-invasive, protocol that yields similar results as more complex protocols to elucidate whole body lactate kinetics in healthy volunteers as well as ICU patients. This may be useful for larger studies on lactate metabolism in septic and other patient groups, with the aim to study to what degree high production rates or low clearance rates contribute to hyperlactatemia.

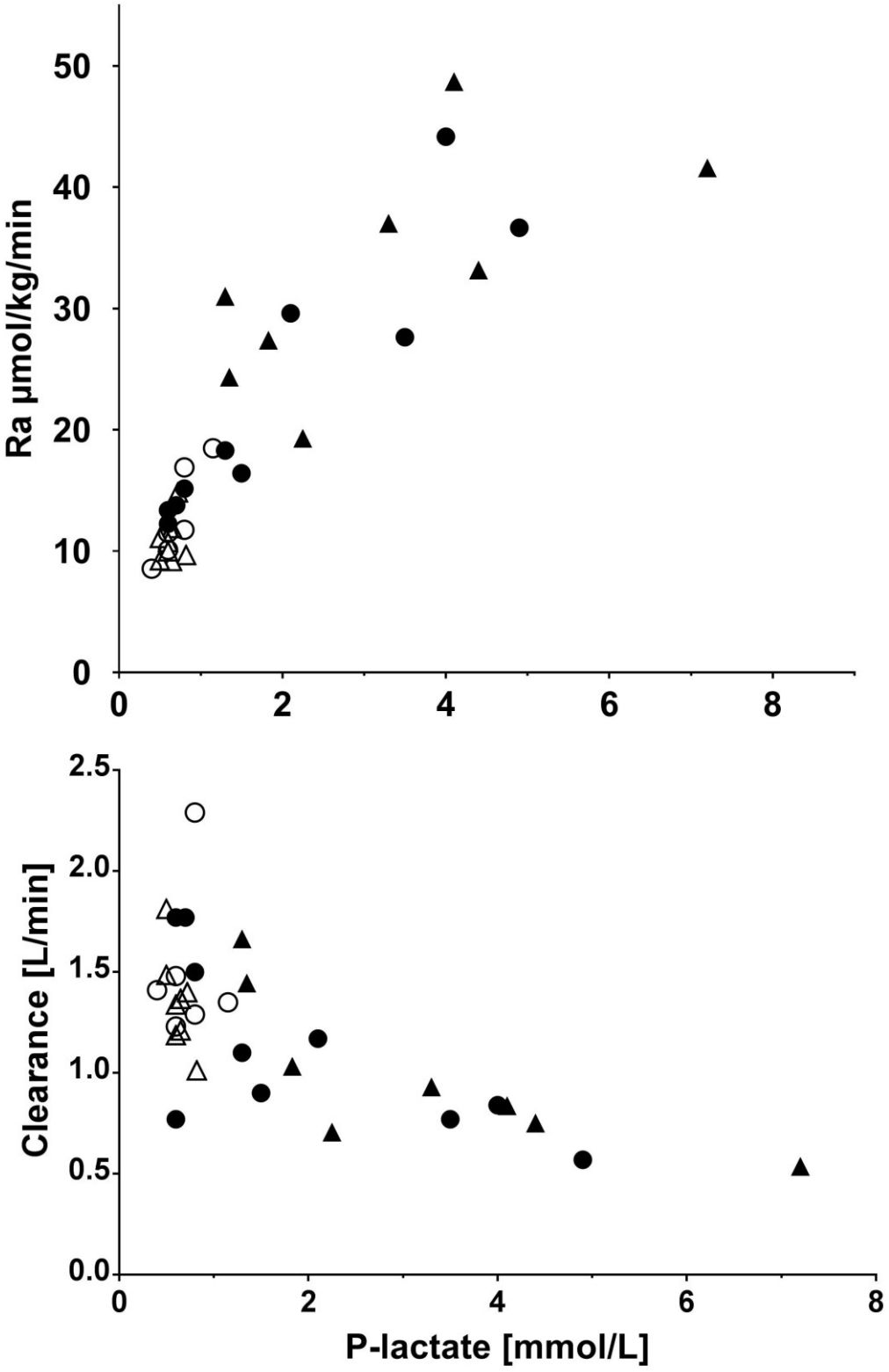

Figure 9. All measurements for whole body rate of appearance (top) and clearance (bottom) from study II (continuous infusion) and IV (bolus injection). *Open triangles* Healthy volunteers during rest study II, *Filled triangles* healthy volunteers under influence of adrenaline. *Open circles* healthy volunteers at rest study IV, *Filled circles* ICU patients study IV

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The mitochondrial derangements seen in severe sepsis and septic shock are still not fully understood. We examined if a factor from plasma of septic patients could directly affect the respiration of isolated mitochondria or in permeabilized muscle fibers, but could find no such effect. This is a complex field and there are a number of different potential mechanisms that could be further examined in the future, such as locally mediated effects or oxidative damage caused by increased ROS formation. Also, failure in the vital organs, like liver, lung, heart and kidney are acutely life-threatening to the patients and preferable the mitochondrial derangements and their possible contribution to the organ failure should be studie¨d in these organs, which in human patients is extremely challenging.

In study III, we retrospectively determined optimal cut-off values for admission plasma lactate and hourly decrease of plasma lactate, to predict mortality in our ICU. Although similar studies have been done previously we introduced a slightly new approach, and whether the hourly reduction rate is clinically useful will have to be determined in future prospective trials. The most interesting was that we studied a patient population that would not necessarily behave in this way, and characterized the patient population in our ICU, which will be used for designing our upcoming studies in septic shock patients. Based on these results we will most likely study lactate kinetics in patients groups based on the defined cut-off values in the future.

Within this thesis we explore two different approaches of using labeled lactate to study lactate kinetics in critically ill patients. The continuous infusion approach with sampling in artery and femoral vein allows for quantification of lactate uptake and release from skeletal muscle in addition to whole body calculations. This method yields more information and is applicable for more detailed physiological studies in fewer subjects, especially when the contribution of skeletal muscle is of interest. Theoretically, this approach will work in other tissue beds as well but the access of the venous drains might be challenging in a critically ill patient population. The method is also useful in cases where effects of an intervention is of interest. It is limited in clinical studies on account of being more invasive due to the need for additional central lines. It might also not be guaranteed that enrichment keeps in steady state throughout the experiment in the case of unstable physiology, which could cause difficulties in infusions and priming doses. The blood flow measurements needed for this approach, requires specific competence and are somewhat user dependent. We also fear that occlusion plethysmography may be unsuitable in hemodynamically unstable patients and plan to perform future studies using Doppler ultrasound.

We also investigated if a bolus approach to administer labeled lactate was feasible and accurately described whole body lactate kinetics. Our method yielded similar results as other methods in healthy volunteers, which in turn were similar to ICU patients with normal

plasma lactate. The notion that no extra invasive procedures or hemodynamic measurements are needed makes it possible to be completed within a short period of time by a single investigator. Since number of samples could be decreased to 14 without sacrificing accuracy, this protocol is easy, minimally invasive and useful to study lactate kinetics on whole body level on larger populations.

As a next step we aim to use this latter protocol to study lactate kinetics in larger cohort of ICU patients with septic shock. Ethical permission is granted and a trial is planned to start during 2019.

Previously, plasma lactate has been mostly studied in septic patients, but increased concentration of (and failure to normalize) plasma lactate have also been shown to correlate with poor outcome in e.g. traumatic brain injury (156) and cardiac arrest (157). These conditions would be of interest to study in more detail in the future, as well as a number of other aspects of lactate kinetics in hospitalized patients. The main aim of these studies would be to better elucidate the roles of impaired production and/or clearance in the different clinical situations with high plasma lactate related to poor outcome.

One such example is sterile inflammation, such as pancreatitis. Others are postoperatively to major abdominal surgery. Both pancreas and esophageal surgery often have increased lactate concentrations during the first night after surgery, but the genesis and predictive value of this is not studied. Elevated lactate after liver surgery are somewhat more studied, but could be caused by different mechanisms, such as decreased clearance due to a compromised liver function.

Today, elevated lactate is recognized as one of the best predictors for in-hospital mortality and is used as a screening tool in emergency departments. A patient presenting with elevated lactate mandates swift assessment by a physician, often leading to immediate resuscitation. One of the great challenges is to examine whether there are patients who are unable to react with lactate elevation, and their need for immediate care thereby underestimated (similar to afebrile septic patients receiving antibiotics later, although being more likely to die from the condition (42)).

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Septisk chock är ett tillstånd där immunförsvarets reaktion mot en infektion leder till akut försämrad funktion i ett eller flera organ. Detta är ett mycket kritiskt tillstånd och en av de vanligaste dödsorsakerna på sjukhus över hela världen. Kliniskt är septisk chock ofta associerat med förvirring, hög puls, lågt blodtryck och minskad urinproduktion. Ofta krävs behandling med stora mängder vätska och potenta läkemedel på en intensivvårdsavdelning (IVA), men även vid sådan vård har septisk chock en dödlighet runt 40%.

Det är fortfarande okänt varför de olika organen sviktar vid sepsis, men eftersom flera organ är påverkade samtidigt misstänker man att det kan bero på en gemensam, central, faktor. Bland annat är kroppens ämnesomsättning kraftigt påverkad på flera sätt. Dels har cellernas ”energifabriker”, mitokondrierna, i olika organ visat sig vara påverkade vid djurstudier, dels har man sett förhöjda nivåer av laktat (mjölksyra) i blodplasma. I den här avhandlingen har vi undersökt om någon faktor i plasma från septiska patienter kan påverka mitokondrier direkt samt studera förändringshastigheten på laktatnivåer hos intensivvårdspatienter med septisk chock närmare. Vi har även undersökt två metoder för att studera laktatmetabolismen (både produktion och konsumtion) hos intensivvårdspatienter.

Studie I: I denna studie isolerade vi fram mitokondrier från skelettmuskulatur hos råttor. Dessa mitokondrier inkuberades sedan med antingen plasma från patienter med septisk chock eller matchade kontroller som nyligen genomgått mellanstor kirurgi. Efter inkubationen analyserades syrekonsumtionen hos mitokondrierna. Vi kunde inte se någon skillnad mellan dessa grupper och därför gick vi vidare med ytterligare ett försök där vi istället inkuberade (permeabiliserade) muskelceller från råttor med plasma från patienter med septisk chock eller friska kontroller före vi analyserade syrekonsumtionen. Inte heller här såg vi någon skillnad mellan grupperna. Vi drar därför slutsatsen att plasma från patienter med septisk chock inte direkt påverkar syrgaskonsumtionen hos isolerade mitokondrier från råtta.

Studie III: Det är sedan länge känt att höga laktatnivåer i plasma korrelerar med hög dödlighet vid sepsis, men också att en oförmåga att snabbt minska dessa nivåer har ett ännu starkare samband med dödlig utgång. I den här studien undersökte vi i mer detalj hur dessa samband ser ut bland patienterna med septisk chock på IVA på Karolinska Universitetssjukhuset i Huddinge. Vi gick igenom samtliga patienter som vårdats för septisk chock på IVA under åren 2015 och 2016. De som hade plasma laktat >2mmol/L samt behov av behandling med noradrenalin (kärlsammandragande läkemedel) inkluderades (sammanlagt 104 patienter). Samtliga deras analyser av laktatvärden under de första 24 timmarna av vård eller fram till att laktatvärdet normaliserades (<1.5mmol/L) undersöktes. De optimala värden för att förutsäga dödlig utgång var >4 mmol/L vid ankomst till IVA, eller en sänkning på mindre än 2.5% per timme.

Studie II och IV: Eftersom omsättningen av laktat vid svår sjukdom är komplex ville vi undersöka två olika metoder för att studera metabolismen av laktat (både produktion och

konsumtion). För att göra detta använde vi oss av isotopmärkt laktat, där en av kolmolekylerna är något tyngre (^{13}C) jämfört med den vanligaste kolisotopen (^{12}C). Detta laktat omsätts i kroppen på samma sätt som kroppseget laktat men kan särskiljas vid laboratorieanalys. Genom att mäta relationen mellan de två formerna av laktat kan man bestämma hur mycket laktat som bildas och konsumeras i kroppen, eftersom man vet hur mycket av det märkta laktatet som givits.

I den första av dessa studier ville vi använda en kontinuerlig infusion av märkt laktat samt titta närmare på muskulaturens omsättning av laktat, då denna verkar vara viktig vid kritisk sjukdom. För att höja laktatnivåerna i plasma använde vi oss av adrenalin. Först genomförde vi en pilotstudie på fyra friska volontärer för att undersöka hur höga laktatnivåer som uppnåddes vid olika nivåer av adrenalintillförsel. Till huvudstudien fick sedan åtta friska volontärer en venös samt en arteriell infart i vardera arm, samt en infart i lårvenen på höger sida. Efter att blodprover tagits påbörjades infusionen av ^{13}C-lakatat som pågick genom hela försöket. Efter två timmar togs blodprover samt ett vävnadsprov från lårmuskeln, samtidigt som blodflödet i benet mättes. Efter det startade en adrenalininfusion som pågick i tre timmar innan provtagningen upprepades. Adrenalinet höjde nivåerna av laktat i plasma (från 1 till 4 mmol/L), muskulaturen i benet ökade sin laktatfrisättning och bidrog till laktatstegringen. Benets laktatomsättning beskrevs bra genom att jämföra arteriella och venösa prover, men muskelproverna hade för stor variation för att tillföra ytterligare information. I det protokoll vi senare ska använda för att studera laktat hos septiska patienter kommer vi därför att avstå från detta. Ett intressant och något oväntat fynd var att adrenalintillförseln ökade den maximala syrekonsumtionen (state 3 respirationen) hos mitokondrierna med 30%.

I den sista studien försökte vi istället undersöka ett mindre invasivt protokoll för att studera laktatomsättning på helkroppsnivå, som helst ska kunna utföras av en enda person och utan att påverka pågående vård. I ett första steg fick sex friska volontärer en bolusdos av ^{13}C-laktat. Därefter togs 43 blodprover under två timmar. Sedan utfördes samma protokoll på tio IVA-patienter. Protokollet gav liknande värden för laktatproduktion som tidigare publicerade studier. IVA patienterna med lågt laktat hade liknande metabolism som de friska volontärerna. Simuleringar visade att antalet prover kan minskas från 43 till 14 (dvs en provtagningsvolym på ca 30 ml) med samma precision.

Våra slutsatser är att protokollet med kontinuerlig infusion ger mer information men är så pass mycket mer invasivt och kräver specialkompetens att det sannolikt är mest användbart för mindre fysiologisks studier. Protokollet med bolusdos ger däremot mindre information men kan med fördel användas i större patientgrupper. Vi kommer härnäst gå vidare med att studera IVA-patienter med septisk chock med detta protokoll. Det finns dock även fler patientgrupper som kan vara intressanta att studera i framtiden, såsom patienter som vårdas efter hjärtstopp, traumatisk hjärnskada eller nyligen genomgått större kirurgi.

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