MIND THE MITOCHONDRIA

M.A. WEFERS BETTINK

Mind the Mitochondria!

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Mind the Mitochondria!

Denk aan de mitochondrieën!

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Chapter 1

Preface and outline

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Disclaimer: the first 4 paragraphs of the introduction were earlier published; Wefers Bettink MA, Arbous MS, Raat NJ, Mik EG. Mind the Mitochondria! J Emerg Crit Care Med 2019;3:45.

A primary goal in the care for critically ill patients is safeguarding an adequate oxygen transport to organs and tissues is. Over the last two decades it has become clear that in certain pathophysiological circumstances macrocirculatory derailment is followed, or accompanied, by microcirculatory dysfunction.[1] Resuscitation strategies to restore and optimize blood flow to organs are based on the idea that restoring oxygen supply will re-establish aerobic metabolism and lead to "healthy parenchymal cells".[2] However, mitochondrial damage and subsequent dysfunction, or cellular adaptation to hypoxia, might attenuate or even counterbalance the positive effects of resuscitation on the cellular level.[3,4]

Mitochondria are the primary consumers of oxygen and the ultimate destination of approximately 98% of oxygen reaching our tissue cells. Most of the oxygen is used for energy production by forming adenosine triphosphate (ATP) through the process of oxidative phosphorylation, but a small amount is used for generating reactive oxygen species and creating heat .[5] While ATP production is the best known function of mitochondria, they also play a key role in calcium homeostasis and cell-death mechanisms. Oxidative phosphorylation has a very high affinity for oxygen and functions well at very low oxygen levels. However, cellular respiration does adapt to changes in oxygen availability at physiological levels, a mechanism known as "oxygen conformance".[6,7]

Oxygen conformance, mitochondrial damage by certain hits (e.g. toxins and medication), mitochondrial dysfunction and autonomic metabolic reprogramming are all factors in sepsis that could contribute to what is known as "cytopathic hypoxia".[8,9] This concept describes insufficient oxygen metabolism in cells despite sufficient oxygen delivery. Altered cellular oxygen utilization and thus reduced oxygen demand could in itself cause decreased microcirculatory blood flow, making microcirculatory dysfunction in sepsis under some circumstances a possible epiphenomenon.[10] Resuscitation and forced restoration of microcirculatory flow could lead to relative hyperoxia, and be counterproductive by increasing reactive oxygen production and intervening with protective adaptation mechanisms.

The complex pathophysiology of a critically ill patient, especially in severe sepsis and septic shock, requires a multilevel approach. In attempting to understand the interplay between macrocirculation, microcirculation and parenchymal cells, the mitochondria are key players that should not be overlooked. We see substantial progress in the development of technologies to assess aspects of mitochondrial function at the bedside, for example direct measurement of mitochondrial oxygen tension and oxygen consumption.[11,12]

Measurement of mitoPO$_2$ and mitoVO$_2$

The method used to measure mitoPO$_2$ and mitoVO$_2$ is described in detail by Mik et al. [11,13] In short, protoporhyrin IX (PpIX) is the final precursor of heme in the heme synthetic pathway. PpIX synthesis takes place in the mitochondria and aminolaevulinic acid (ALA) is the rate limiting product in this process. ALA administration leads to a substantial enhancement of PpIX concentration in the mitochondria. PpIX possesses a triplet state that reacts strongly with oxygen, making its delayed fluorescence lifetime oxygen-dependent according to the Stern-Volmer equation.[14]

MitoVO$_2$ is measured directly after local occlusion of the microcirculation and thus stopping oxygen supply, this is obtained by local pressure on the measurement probe. This simple procedure of stopping oxygen supply creates reproducible stop flow conditions and therefore reproduceable mitoVO$_2$ measurements. The first implementation of this technique was described by Harms et al.[15]

Whilst it might be relevant to measure mitochondrial oxygenation and oxygen consumption in sepsis, no golden standard for these measurements exists. In fact, most mitochondrial function tests are invasive or can only be performed in a research setting (NMR and NADH fluorometry). In this thesis, we present the translational journey of a Cellular Oxygen METabolism monitor (COMET) from an animal model to the patient with sepsis. We show the possibility of bedside monitoring of mitochondrial oxygenation (mitoPO$_2$) and mitochondrial oxygen consumption (mitoVO$_2$) for a patient with sepsis.

Outline

This thesis is divided in 3 parts. Part one focusses on the development of the COMET monitor and its calibration in humans. Part two focusses on the changes in mitochondrial function in a sepsis like experimental model (rat or human volunteers) or during sepsis (patients). Part three focusses on perioperative use of the COMET monitor as a parameter of cellular oxygenation.

In **chapter 2** we give an overview of the physiology of mitochondrial function and adaptation. We describe possible causes of mitochondrial dysfunction, the concept of cytopathic hypoxia, the loss of hemodynamic coherence and ways to assess aspects of mitochondrial function in patients.

Part one

In part one we answer the research questions: (i) are we able to measure changes in mitochondrial oxygenation in the human skin with the new COMET monitor, and (ii) how do the measurements of the COMET monitor relate to traditional methods of measuring cellular oxygenation?

In **chapter 3** we give a description of the COMET monitor. We discuss the output of the monitor and the first human data of perioperative use of the monitor during neurosurgery. In the case described, the COMET monitor was able to measure a decline in mitochondrial oxygenation, whereas the O2C monitor (a monitor for observing tissue oxygenation and measuring microcirculation) was not able to detect a change in tissue oxygenation. This is the first indication of the potential accuracy of the COMET monitor in specifically detecting subtle changes in mitochondrial oxygenation.

In **chapter 4** we describe calibrating the COMET monitor in human skin. In the same experiment, we directly compare different methods to measure oxygen metabolism. Although the technique of the COMET monitor has extensively been calibrated in cell cultures, organs, a rat model and healthy volunteers, the final monitor had not yet been calibrated in human use. Furthermore, we compare how the different methods and measurement sites react to stop flow conditions and respond to the dynamic measurement of mitoVO$_2$ by the COMET monitor.

Part two

In part two we answer the questions: (i) are we able to measure changes of mitochondrial function in a rat model of sepsis and how do these changes translate to an identical model in human volunteers, and (ii) are we able to measure these changes in mitochondrial function in ICU patients with sepsis?

In **chapter 5** we compare the non-invasive COMET technique to asses mitochondrial function with the most commonly used *ex vivo* technique based on isolated mitochondria from muscle biopsies using a Clark-type oxygen electrode. A secondary goal is to stop the

diminutive effect of our endotoxemia model on mitochondrial function by administration of succinate. Aerobic metabolism, a primary function of the mitochondria, is enabled by the electron transport chain. The electron transport chain has two possible electron donors of which NADH via complex 1 is the dominant pathway. Our endotoxemia model is known to create dysfunction of complex 1. Therefore, administration of succinate, the electron donor for complex 2, might be a possible therapy for the altered mitochondrial function in our endotoxemia model.

In **chapter 6** we assess whether the measured changes in mitoPO$_2$ and mitoVO$_2$ during an endotoxemia model in rat translates to a human volunteer endotoxemia model. In the Radboud University experimental Intensive Care Department, human endotoxemia models are based on the same method described in chapter 5. In a collaboration between our laboratory and the experimental ICU of the Radboud UMC, we performed the first ever endotoxemia experiment, monitoring mitochondrial function on the bedside with our COMET technique.

In **chapter 7** we measure changes in mitochondrial function in ICU patients with sepsis. Bedside measurements were obtained by assessing mitochondrial function parameters (COMET) and microcirculatory parameters (O2C). Off-site mitochondrial function was measured ex vivo in platelets and mitochondrial damage was assessed by determining mitochondrial DNA in plasma.

Part three

In part three we answer the questions: (i) are we able to measure stable values of mitoPO$_2$ in adults during neurosurgery in stable operating conditions, and (ii) are we able to measure alterations in mitoPO$_2$ during high risk neonatal surgery?

Measurement of mitochondrial oxygenation is possible with the COMET monitor. Perioperative dynamics of mitochondrial oxygenation is a promising tool for anesthesiologists to monitor oxygen delivery and intervene when required. The first feasibility of the COMET monitor during anesthesia and brain surgery is described in **chapter 8.** The first use of the COMET monitor in high risk neonatal surgery is described in **chapter 9.**

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Chapter 2

Mind the mitochondria!

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Abstract

Safeguarding an adequate oxygen transport to organs and tissues is a prime goal in the care for critically ill patients. Over the last two decades it has become clear that in certain pathophysiological circumstances macrocirculatory derailment is followed, or accompanied, by microcirculatory dysfunction. Resuscitation strategies to restore and optimize blood flow to organs are based on the idea that restoring oxygen supply will re-establish aerobic metabolism and lead to "healthy parenchymal cells". However, mitochondrial damage and subsequent dysfunction, or cellular adaptation to hypoxia, might attenuate or even counterbalance the positive effects of resuscitation on the cellular level. In this short review we will address mitochondrial function and adaptation, causes of mitochondrial dysfunction, the concept of cytopathic hypoxia, (loss of) hemodynamic coherence and ways to assess aspects of mitochondrial function in patients.

Mitochondria are the primary consumers of oxygen and the ultimate destination of approximately 98% of oxygen reaching our tissue cells. Most of the oxygen is used for energy production by oxidative phosphorylation, but a small amount is used for generating reactive oxygen species and heat generation. While adenosine triphosphate (ATP) production is the best known function of mitochondria, they also play key roles in calcium homeostasis and cell-death mechanisms. Oxidative phosphorylation has a very high affinity for oxygen and functions well at very low oxygen levels. However, cellular respiration does adapt to changes in oxygen availability at physiological levels, a mechanism known as "oxygen conformance".

Oxygen conformance, mitochondrial damage by certain hits (e.g. toxins and medication), mitochondrial dysfunction and autonomic metabolic reprogramming are factors that could contribute to what is known as "cytopathic hypoxia". This concept describes insufficient oxygen metabolism in cells despite sufficient oxygen delivery in sepsis. Altered cellular oxygen utilization and thus reduced oxygen demand could in itself cause decreased microcirculatory blood flow, making microcirculatory dysfunction in sepsis under some circumstances a possible epiphenomenon. Resuscitation and forced restoration of microcirculatory flow could lead to relative hyperoxia, and be counterproductive by increasing reactive oxygen production and intervening with protective adaptation mechanisms.

The complex pathophysiology of a critically ill patient, especially in severe sepsis and septic shock, requires a multilevel approach. In understanding the interplay between macrocirculation, microcirculation, and parenchymal cells the mitochondria are key players that should not be overlooked. Progress is being made in technologies to assess aspects of mitochondrial function at the bedside, for example direct measurement of mitochondrial oxygen tension and oxygen consumption.

Introduction

In critical illness, one of the mainstays of therapeutic interventions is to safeguard adequate oxygen transport to organs and tissues. While in the past the focus mainly was on optimizing macrohemodynamic variables, over the last two decades, the microcirculation has become a prime subject of research and clinical thinking. The dysregulated oxygen transport to tissue in states of shock and resuscitation and the needed interventions to reverse them are still a topic of controversy. Current evidence shows that not a deterioration of systemic variables per se but rather a failure of the microcirculation to transport oxygen to parenchymal cells is the cause of circulatory compromise. Pathological heterogeneity of microcirculatory perfusion leading to functional shunting in the microcirculation causes local hypoxia and reduced cellular respiration, macroscopically manifesting as a reduction in oxygen extraction (1,2).

The defect in oxygen extraction observed during states of sepsis and shock is not only due to alterations in microcirculatory function. In some cases, resuscitation in terms of restored macrohemodynamic and microcirculatory parameters does not lead to clinical improvement and indirect indications of tissue hypoxia, such as high serum lactate, remain. Reduced cellular oxygen consumption, i.e. caused by mitochondrial dysfunction, could mimic such states. Cellular metabolic adaptation could also lead to reduced oxygen extraction and subsequently to a reduced ability of the mitochondria to produce ATP. It is not even unthinkable that under specific circumstances an apparent microcirculatory dysfunction might be an epiphenomenon caused by an in itself adequate adaptation of the microcirculation to an adapted or failing utilization of oxygen and nutrients by parenchymal cells. In any case, mitochondrial alterations in states of shock and sepsis have long been observed (3). If reduced mitochondrial oxygen consumption, either caused by mitochondrial dysfunction due to direct damage or as part of an adaptation mechanism, exists in the presence of normal microcirculatory oxygen transport, then this would have a dramatic impact on the current understanding of resuscitation medicine. Recently, evidence that this might be the case is emerging from both animal and clinical studies. Current resuscitation is entirely focused on promoting oxygen transport to tissues via convection and passive diffusion (4).

Research on the microcirculation in critical illness has been greatly boosted by the emergence of bedside tools that allow direct visualization of the microcirculation of a patient, for example under the tongue (5,6). To further unravel the pathophysiological mechanisms and better understand the interaction between microcirculatory impairment

and alterations at the cellular and mitochondrial level we need tools that allow us to look beyond the microcirculation, probing directly aspects of cellular metabolism. Ultimately, the clinician should have access to bedside tools that enable monitoring and verification of treatment success at the microcirculatory, cellular and mitochondrial level. Such tools are currently being developed and tested both in the laboratory and in clinical trials. In this review, we will briefly discuss mitochondrial function, adaptation, causes of dysfunction, the concepts of cytopathic hypoxia and loss of hemodynamic coherence. We will briefly discuss ways to look at the mitochondria in the clinical setting, with a special focus on the novel COMET device. The COMET allows, at the bedside, direct measurement of mitochondrial oxygenation and respiration by an optical technique.

Aspects of mitochondrial function

Mitochondria are double-membrane organelles found in almost all cell types, with the exception of erythrocytes. One of the main functions of mitochondria is to generate adenosine triphosphate (ATP) through oxidative phosphorylation. Over the last two decades, mitochondrial research has undergone a renaissance. Apart from its role in cell bioenergetics, a whole series of discoveries revealed mitochondrial roles in cell death, disease pathology, aging, thermogenesis, oxidative stress, cell signaling and cellular regulation. A review series about the role of mitochondria in aging and various pathophysiological states has been recently published elsewhere(1). The following paragraphs will only provide a short overview of mitochondrial function.

Mitochondria are the primary consumers of oxygen and are responsible for approximately 98% of total body oxygen consumption. Oxygen is ultimately used at complex IV of the electron transport chain in the inner mitochondrial membrane. Reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), generated in the Krebs cycle, are transferred from carrier molecules to the electron transport chain on complex I and II respectively. The resulting electron transport through the chain causes protons to be pumped to the intermembrane space. This proton pumping causes an electrochemical potential over the inner membrane that is used to convert adenosine diphosphate (ADP) to ATP by ATP synthase. ATP is the energy currency of the cells and used for driving cellular processes like maintaining membrane potentials, protein synthesis and replication.

The coupling of mitochondrial respiration to ATP production is not 100% and this leads in part to oxidative phosphorylation generating not only ATP but also reactive oxygen

species (ROS). Mitochondria-generated ROS were originally considered potentially toxic by-products, but are now considered to be key signaling molecules in various (patho) physiological processes. For example, mitochondrial ROS at low levels causes metabolic adaptation in hypoxia while high levels of mitochondrial ROS activate apoptosis (3) and autophagy (4). Thus, mitochondrial ROS are associated with processes ranging from cellular adaptation to inducing cell death. Also, mitochondrial ROS are involved in regulating the inflammatory response and can be stimulated by danger signals. An example is the activation of Toll-like receptors (5) by bacterial ligands such as lipopolysaccharide (6).

While most mitochondrial oxygen consumption is used for generating ATP, mitochondrial oxidative metabolism is not limited to ATP generation. As stated above, a small amount of oxygen consumed is directed toward ROS production. Furthermore, a certain amount of respiration is uncoupled from ATP production and lost as heat (7) and used for thermogenesis (8). The amount of mitochondrial coupling varies from tissue to tissue and is typically high in heart and much lower in liver and skeletal muscle (9). Specific uncoupling proteins within the inner mitochondrial membrane use the mitochondrial membrane potential for thermogenesis and are present in e.g. brown adipocytes.

Next to functions linked to the respiratory chain, recent discoveries point to a much broader role of mitochondria in cell homeostasis. For example, mitochondria possess a calcium uniporter (10,11)and might play a role in intracellular calcium homeostasis (12). Also, while mitochondria are essential in sustaining life, they play a major role in (programmed) cell death. Opening of the mitochondrial permeability transition pore, as a result of a stressful stimulus such as calcium or ROS overload, leads to loss of the mitochondrial membrane potential (13). The collapse of the membrane potential results in ATP depletion and necrosis (14), and the release of mitochondrial content such as cytochrome c leads to apoptosis (15).

The role of oxygen

Molecular oxygen is a diatomic gas that constitutes approximately 21% of the volume of air. The electron configuration of the oxygen molecule has two unpaired electrons with the same spin in degenerate orbitals. Therefore, oxygen is paramagnetic and the ground state of oxygen is a triplet state, which is very unusual compared to many

other biochemically relevant molecules. The ground state is a triplet state that has the important implication that molecular oxygen does not react directly with many other molecules, in contrast to the highly reactive oxygen radical singlet oxygen.

The adequate supply of oxygen to organs and tissues is of pivotal importance to sustain mammalian life. Aerobic metabolism is maintained through inhalation of air in the lungs and subsequent transport of the absorbed oxygen to tissues via the circulating blood. The flow of hemoglobin-bound oxygen through the macro- and microcirculation and diffusion of molecular oxygen into the tissue cells brings oxygen to its ultimate destination, the mitochondria. In the mitochondria, oxygen is used in oxidative phosphorylation in order to efficiently produce adenosine triphosphate (ATP) that acts as the energy source for many cellular processes. Oxygen also plays a role in many other biochemical processes and mammalian tissue contains a large number of oxygen-consuming enzymes (7), for example for reactive oxygen species generation in signal transduction (8,10).

Cellular hypoxia causes mitochondrial dysfunction, oxidative damage, activation of inflammatory cascades and complement activation, ultimately leading to tissue death. If oxygenation is restored after a prolonged period of time hypoxia, re-oxygenation injury, or ischemia-reperfusion injury, occurs which leads to augmented oxidative stress and tissue injury (11,12).

In this respect it is interesting to note that it is actually unknown, or ill-defined, what "hypoxia" actually is. For example, from a medical point of view one could define hypoxia as a pathological condition in which the body or a region of the body is deprived of an adequate oxygen supply. But the question here obviously is: what does adequate mean in this respect? In the classical view, cellular respiration is unaffected by oxygen levels until PO_2 decreases below 2 to 3 mmHg (13-16)**.** This is because of the high affinity of the mitochondrial respiratory chain for oxygen. In the more modern view, oxygen availability is directly influencing cellular metabolism and function by processes like oxygen conformance and cellular metabolic reprogramming, processes that appear to occur at much higher oxygen levels (see below). So, while in the classical view hypoxia is almost equivalent to anoxia, in the modern view any perturbation from a current PO_2 level could alter mitochondrial respiration and ultimately cellular function. Therefore, the actions of medical doctors to achieve "adequate tissue oxygenation" might in the end, unintentionally, alter cellular metabolism and function due to alterations in tissue oxygen levels, e.g., by superfluous oxygen administration or vasopressor therapy (17).

Metabolic adaptation

Two important metabolic adaptation mechanisms that may protect a cell from an energy crisis due to reduced oxygen delivery have been described in literature. One is a metabolic shift from aerobic oxidative phosphorylation to an-aerobic glycolysis also referred to as metabolic reprogramming (18). The other is oxygen conformance, which Arthur et al. (19) described as "the ability to reduce energy demand, and hence oxygen consumption, in response to a decline in oxygen availability without a decrease in the concentration of ATP". Although it is likely that both mechanisms play a role in the adaptation to the reduced supply of oxygen, here the focus will be on oxygen conformance.

Although the mechanism of oxygen conformance was already mentioned by Peter Hochachka in 1986 (20) it was not until 1993 that Schumacker et al. (21) described a decrease in oxygen consumption in primary rat hepatocytes in response to moderate oxygen (20-50 mmHg) deprivation. They showed that the duration of hypoxia and the time period in which oxygen levels were decreased was important for the cellular response, i.e. whether the cells reduced their oxygen consumption rate or not. Before their report, studies had always used a quick depletion of oxygen usually for a short period (several minutes) and shown that the rate of oxygen consumption was oxygen-independent till a PO_2 of a few mmHg (22,23). Schumacker et al. (21) confirmed these previous results and showed that in primary rat hepatocytes in which PO_2 was rapidly reduced (100 to 0 mmHg in < 40 min, oxygen consumption did not change till PO_2 was less than 10 mmHg and ATP levels were preserved. In contrast, when PO_2 levels were slowly reduced (> 2 hr) changes in oxygen consumption already occurred around 70 mmHg with a ~45% decrease in oxygen consumption around 15 mmHg. ATP levels followed the drop in oxygen consumption while NAD(P)H levels increased compared to rapidly deoxygenated cells.

At low O_2 tensions of around 20 mmHg the reduced oxygen consumption could be maintained for 18 hours without a decrease in cell viability. The fact that cell viability was preserved during prolonged hypoxia in the slow hypoxia group indicates that the balance between ATP production and consumption was maintained and therefore the cells use less ATP. In a follow-up study Subramanian et al. (24) measured the changes in metabolic demand for ATP during oxygen conformance. They hypothesized whether viability was maintained by preferentially suppressing non-essential processes while leaving essential processes involved in cell homeostasis intact. They confirmed their hypothesis by showing that the non-essential processes of ATP-dependent glucuronidation and sulfation of

acetaminophen were decreased significantly during hypoxia. At the same time Na^+/K^+ pump activity, an essential process for maintaining cell ion homeostasis was maintained. No lactate production was measured during prolonged moderate hypoxia which supports the finding that cellular ATP demand was down-regulated by the reduction of non-essential metabolic processes. Later studies also described oxygen conformance in other cell types such as skeletal muscle (25) and chick cardiomyocytes (26).

The mechanism of oxygen conformance is only partly known. Cytochrome c oxidase (complex 4), the terminal electron acceptor in the electron transport chain, is reversibly inhibited after expose to low oxygen concentration (< 50 mM) for several hours (27). It has been suggested that cytochrome C oxidase could act as an oxygen sensor as its reduction state has an effect on its kinetic activity (28) and hence oxygen consumption by the cell. A later study in cardiomyocytes confirmed this mechanism (29). The mechanism involved in the subsequent reduction in ATP as described by Subramanian is less clear. It might involve a difference in ATP affinity of ATP consuming proteins (24). Alternatively AMPK, a kinase that maintains a balance between cellular ATP generation and consumption could be involved (30). It has been shown that AMPK becomes activated during hypoxia and is dependent on mitochondrial complex III (31). It is expected that other causes of ATP reduction, like mitochondrial dysfunction, activate similar pathways to reduce ATP demand.

Hits on mitochondria

Next to their role in cellular adaptation and the more indirect downregulation of mitochondrial respiration, mitochondria are also directly susceptible to certain “hits” that cause mitochondrial damage and dysfunction as part of pathophysiology and treatment. Unfortunately, mitochondrial (dys)function is difficult to measure in the ward and as a consequence there is little clinical awareness of its occurrence. However, general goals in medicine, such as securing oxygen supply, starting therapy for infectious disease, treating cancer, performing an operation and securing comfort during the operation are likely to influence mitochondrial function. For example, acute changes in mitochondrial function might be a cornerstone in the development of SIRS or sepsis. The accompanying mitochondrial dysfunction might be caused by a combination of hyperoxia/hypoxia, medication and inflammation-induced metabolic changes, as shown in figure 1. A short overview of possible hits on mitochondria follows.

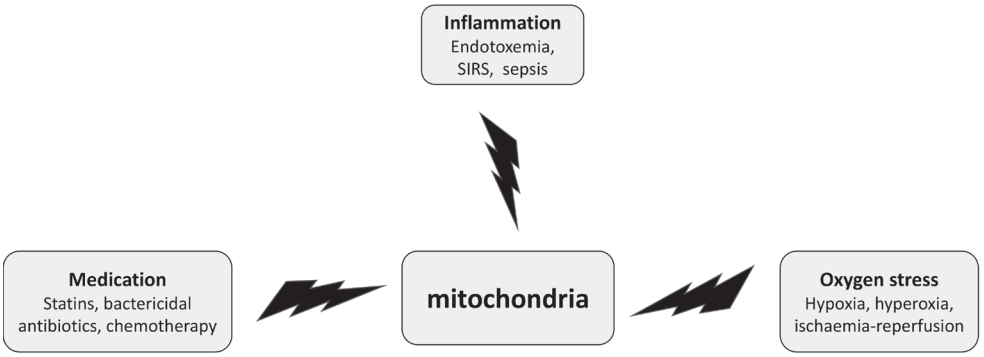

Figure 1. Hits on mitochondria. Different hits on mitochondria which might be present for a patient.

A long standing standard practice on the ward of liberally supplying oxygen to patients led to rise in mortality (32). Setting a more conservative goal for intensive care patients with a saturation between 94 and 98% decreased such negative effect of superfluous oxygen administration (33). Furthermore hyperoxia led to mitochondrial and cellular dysfunction (34). Mitochondria also adapt to hypoxia with a reduction of metabolism (35). Even return of cellular oxygenation after hypoxia induces cellular damage. Reperfusion after cardiac ischemia leads to mitochondrial damage and might be as damaging to cells as the ischemia alone (36).

Medical treatment of diseases or secondary prevention may also lead to mitochondrial side effects. Indeed, in recent years new proof demonstrated the role of mitochondrial damage and/or dysfunction as central players in these side effects. Statins are a typical example of a widely-used drug causing mitochondrial side effects. The basis of preventing cardiovascular disease is lowering of LDL cholesterol, usually successfully achieved by prescribing such statins. However, 5-29 % of the patients stop the use of statins due to side effect of statin- induced muscle disease. Current evidence points to mitochondrial dysfunction as an important factor, but the exact process has not been fully understood (37). Many drugs used in the operating theater and in the ICU are also known for being able to induce mitochondrial alterations, for example propofol. During prolonged sedation a well-known side effect of this drug is the propofol-infusion syndrome; usually characterized by an unexplained high anion gap, metabolic acidosis, rhabdomyolysis and hyperkalemia. Propofol-infusion syndrome is a rare but serious complication, with a recent recommendation to check for mitochondrial disease if this complication develops (38-40). Other drugs commonly encountered in the ICU-setting that potentially cause mitochondrial side effects are antibiotics and chemotherapeutics. Serious side effects,

e.g. ototoxicity, are known from bactericidal antibiotics and are linked to mitochondrial dysfunction (41). Chemotherapy-induced cardiotoxicity (42) and myopathy (43) are linked to the oxidative stress on mitochondria caused by these therapies. Although most recognized mitochondria-linked side effects by medication are severe, they are not limited to a single medication group and seem to be widespread. It is unknown if and how medication-induced mitochondrial dysfunction might contribute to the complex pathophysiology seen in ICU patients. Small alterations that in the general population are likely to remain subclinical might add to e.g. sepsis-induced metabolic dysregulation and become an important factor in the progression to multi-organ failure.

In translational research on sepsis, several endotoxemia models in rat and pigs lead to the description of the development of mitochondrial dysfunction over time (44-46). Infectious and inflammatory driven changes in mitochondrial function in humans are not a new phenomenon either (3,47). Because of invasiveness, muscle biopsies, needed for the classical measurement of mitochondrial function in isolated mitochondria, are rarely performed in the ICU. However, differences in development of mitochondrial dysfunction in different muscle types have been shown (48). Also a correlation between mortality, severity of organ dysfunction and mitochondrial dysfunction has been demonstrated in several studies (3,49-51). Although a link has been shown between mitochondrial dysfunction and sepsis, a proof of causality is missing and some argue mitochondrial dysfunction in sepsis might be an epiphenomenon (50). Alternatively, mitochondrial dysfunction and a reduced mitochondrial respiration and ATP production have been suggested to mimic hypoxia, so-called “cytopatic hypoxia” (52).

Cytopathic Hypoxia

In intensive care, a primary goal of the intensivist is to preserve organ function or replace it. Corner stones of sepsis treatment on the intensive care are supportive care, safe-guarding a sufficient macrohemodynamic organ perfusion (sufficient mean arterial pressure, lowering of lactate), replacement of organ function (e.g. ventilator support, renal replacement or extracorporal membrane oxygenation), and early antimicrobial therapy. The measurement of microcirculatory function, let alone mitochondrial function, is not part of standard care on the ward and is, as far as we are aware, only performed in research settings. Although lactate is an important factor indicating anaerobic metabolism and therefore tissue hypo-perfusion in sepsis (53), the level of lactate is a crude measure and is known to have pitfalls (54). Cytopathic hypoxia is a concept describing insufficient oxygen metabolism in cells despite sufficient oxygen delivery (55). This apparent tissue

hypoxia might need more complex monitoring than lactate and central venous oxygen concentration to effectively monitor its development and reaction to therapy during sepsis (56).

If an infection produces enough inflammatory molecules to produce a SIRS reaction, the first vital signs of a serious infection include tachycardia and hypotension resulting in shock. Macrohemodynamic collapse is prevented by aggressive fluid resuscitation and circulatory support via noradrenaline. However, monitoring the effect of collapse and resuscitation on microcirculatory or mitochondrial function is not part of the standard of care. The return of microcirculation during the resuscitation of a patient with sepsis improves survival (57). In animal models a clear link is also found between a SIRS reaction and mitochondrial dysfunction,(44,45,58,59) and early mitochondrial dysfunction in sepsis limits the chance of patient survival.(51) Mitochondrial dysfunction in sepsis is not a new concept, as early as in 2002 a direct link between mitochondrial function, organ dysfunction and mortality was described (3).

Aggressive and early fluid resuscitation is one of the pillars of sepsis treatment, with a modest improvement of mortality (60). Mortality is mostly dependent on the amount of organ dysfunction and responsiveness on early treatment goals (60,61). Preserving vital parameters and microcirculatory perfusion during resuscitation only seems to lead to a modest improvement of mortality. Since mitochondrial dysfunction is linked to an increase in mortality, this results in a complex interaction. On one side, the microcirculation is influenced by the mitochondria of the parenchymal cells and on the other side it is affected by the macrocirculation (4). The important question then is whether the (apparent) loss of hemodynamic coherence between the macrocirculation and microcirculation should be treated aggressively by hemodynamic measures or that, alternatively, apparent microcirculatory dysfunction should be seen as an epiphenomenon caused by cellular and mitochondrial issues (dysfunction or adaptation). In the latter case, additional attempts to “optimize” macrohemodynamic parameters and microcirculatory perfusion might prove counterproductive. The answer probably varies in different patients and might even change in single patients over the course of the disease and due to given treatments.

Table 1 The COMET different measurement types.

Single measurement	One measurement per activation of the touchscreen non physical button.
Interval measurement	The COMET will measure in a set interval: At the start of the interval a measurement is done, and the interval time can be chosen (60 min, 20 min, 5 min, 1 min)
Dynamic measurement	The COMET can conduct a series of up to 120 measurements, one measurement per second.

Hemodynamic coherence

Resuscitating critically ill patients from different states of shock is key and remains a challenge in critical care. Currently, the main focus is on administration of fluids and vasoactive medication, targeting the normalization of systemic hemodynamic parameters such as blood pressure, cardiac output and venous saturation. However, many randomized trials have failed to show a consistent difference between patient groups that were compared based on interventions directed at these systemic hemodynamic variables (62-66). This could be due to the trial design and trial execution, the intervention, or the heterogeneity of the population. Alternatively, our interventions aimed at improving cellular oxygen delivery might fail to do so or cells were not able to use the delivered oxygen appropriately. Therefore, to further our knowledge about which intervention most benefits the patient, we will need a more physiological, mechanistic and comprehensive approach.

Shock is a condition in which oxygen delivery to the cells and mitochondria is insufficient to sustain cellular activity and thus organ function. We therefore should add targeting microcirculatory and mitochondrial function to our conventional resuscitation targets (i.e. systemic variables). Particularly, we should study whether the condition of shock is already a condition in which systemic and microcirculatory and cellular measures diverge. We also have to study whether our interventions aimed at improving the systemic hemodynamic parameters of a patient are actually improving or maybe worsening our patients microcirculatory and mitochondrial function. Therefore, we should focus on and study this coherence of the macro- and micro and cellular situation with respect to diagnosis and effect of therapeutic interventions.

Hemodynamic coherence is the coherence between the macrocirculation, microcirculation and the parenchymal cells, in which resuscitation procedures aimed at the correction of hemodynamic variables are effective in correction of regional and microcirculatory

perfusion and oxygen delivery to the parenchymal cells such that they are able to perform their functional activities in support of organ function (67). There are a number of studies that show that improving systemic macrocirculatory parameters does not lead to improvement of the microcirculation (68,69). This lack of coherence was associated with increased morbidity and mortality (68-70). Additionally, we have to focus on the mitochondrial function in the different states of shock. After all, both the coupling of macrocirculation and microcirculation and the coupling of microcirculation and cellular energetics are impaired in the different states of shock and particularly in sepsis. Van Genderen showed that coherence between macrocirculation and microcirculation existed in an obstructive shock model in pigs , but was lost in a septic shock model (71). Also in septic patients loss of coherence was frequently found (70). In a rat endotoxemia model mitochondrial respiration remained reduced by 38 % despite fluid resuscitation restoring mitochondrial oxygenation back to baseline values (45). Such restored oxygenation seems superfluous in comparison to the reduced oxygen demand. Whether this is an example of loss of coherence between cellular oxygen demand and microcirculatory oxygen delivery remains to be clarified.

Furthermore, there are two more dimensions that have to be included in the discussed multidimensional approach. These are the loss of coherence in the different organ systems and the timing of resuscitation and their effect on incoherence and uncoupling. Loss of coherence can vary in the different organ systems. In animal and human studies the intestinal microcirculation particularly remained much longer unresponsive to resuscitation while the macrocirculation and sublingual microcirculation already were restored (72) (73). In septic patients the coherence between macrocirculation and different microcirculatory systems could take up to 3 days to occur (73). Controversially, Ospina-Tascon found that the microcirculation was improved by early fluid resuscitation, but was unaffected by resuscitation later in the course (74). Thus, the exact timing of resuscitation and its effect of coherence and coupling of macrocirculation, microcirculation and mitochondrial function over time remain to be elucidated.

Ways to measure mitochondria and current insights

While there is need to assess mitochondrial function in critically ill patients, existing standard technique are invasive and ex vivo (muscle biopsies) and/or cumbersome (NMR), and not readily available 24/7 in a general hospital. Ex vivo techniques that use tissue biopsies have been mainly used to determine mitochondrial dysfunction in patients. From these tissue biopsies, cells or even mitochondria are isolated before actual

measurement of oxygen consumption can start. Most common techniques measures oxygen consumption using Clark electrodes (75), like the high resolution respirometer (76). Further evaluation of the mitochondrial respiratory chain is possible by measuring individual complex activity or concentration of the complexes in isolated mitochondria (77).

The location of the muscle biopsy maybe important, since a difference was found between leg muscle and diaphragm (48). Measurement of mitochondrial function is possible in isolated platelets or peripheral blood mononuclear cells (78,79). Of essence in measuring mitochondrial function is the choice of buffer in which the cells are measured, since exposing healthy cells to plasma obtained from patients with sepsis lead to mitochondrial dysfunction (80). In platelets early increase of mitochondrial oxygen metabolism has been linked to an increase in mortality (51,81). Since the location of biopsy and the chosen buffer clearly influence the measured mitochondrial function, interpretation of the results found by respirometers might be difficult. Furthermore, measuring mitochondrial function with ex vivo techniques requires special laboratory equipment (82) and trained staff. Both are not available in our general hospitals, which limits this type of monitoring to a research setting in academic hospitals.

Measurement of aspects of mitochondrial function *in vivo* is also possible with Nuclear Magnetic Resonance (NMR)-technique and NADH fluorometry (83,84). With the NMR-technique, phosphorus (P) and its transport in the cell can be measured. phosphorus is used by the cell as an energy source (ATP), and thus metabolic flux can be measured. Since a metabolic flux measurement requires use of ATP, an active muscle is required during the NMR measurement.

NADH fluoroscopy measures the NADH fluorescence at 450 nm (blue light). NADH shows fluorescence after photoexcitation with ultraviolet light, but NAD^+ does not show such fluorescence. Measuring the ratio between the reduced and oxidized form gives a relative redox potential. While this technique works well in a laboratory setting, e.g. in fluorescence microscopy on single cells, it is very difficult to achieve reliable measurements *in vivo*. Both NMR and NADH fluoroscopy measure metabolic state, but the inability to perform bedside monitoring and its costs makes NMR not suitable for standard clinical use in sepsis. NADH fluoroscopy is available for bedside monitoring and changes in metabolic state are possible in a clinical setting (85). However, standard monitoring of mitochondrial function by NADH fluoroscopy is not yet an option due to sensitivity to artifacts and interpretation issues.

COMET (CellularOxygenMETabolism)

In search for bedside *in vivo* real-time monitoring of mitochondrial function the Cellular Oxygen METabolism (COMET) monitor was developed (86). The COMET (Photonics Healthcare B.V., Utrecht, The Netherlands) measures mitochondrial oxygen tension (mitoPO$_2$) with delayed fluorescence of mitochondrial protoporphyrin IX (PpIX) (87). Stopping microcirculatory blood flow by pressing the measuring probe on the skin allows the measurement of the mitochondrial oxygen consumption (mitoVO$_2$), here expressed as oxygen disappearance rate (ODR) (88).The ODR measurement is a non-invasive technique to assess mitochondrial respiration *in vivo*. A first feasibility study with the COMET was performed by our group and mitoPO$_2$ and ODR were measured in healthy volunteers (89). Recently Baumbach et al. showed COMET measurements of mitoPO$_2$, mitoVO$_2$ and mitoDO$_2$ during exercise in healthy volunteers (90). They introduced mitoDO$_2$, a measure for mitochondrial oxygen delivery, as a parameter derived from the dynamics of mitoPO$_2$ during the microvascular reperfusion phase after the release of pressure used for measuring mitoVO$_2$.

MitoPO2 and ODR

The principle of the technique and its development have been described elsewhere (17,87). In short, the COMET monitor uses oxygen-dependent quenching of the delayed fluorescence lifetime of an endogenously synthesized porphyrin, PpIX. Delayed fluorescence of mitochondrial PpIX is a method to measure mitoPO$_2$ in living cells and tissues, non-invasively and feasible in humans. PpIX is the final precursor of heme in the heme biosynthetic pathway. PpIX is synthesized in the mitochondria and administration of 5-aminolevulinc acid (ALA) substantially enhances the PpIX concentration. Photoexcitation of PpIX populates the first excited triplet state, and causes the emission of red delayed fluorescence. The delayed fluorescence lifetime is inversely related to the mitoPO$_2$ according to the Stern–Volmer equation. The background of the delayed fluorescence lifetime technique is extensively described elsewhere (87,91,92).

Clinical example

During a small clinical pilot, part of a larger observational study (IRB approved, CCMO number NL51937.078.15), we measured an example of change in mitochondrial oxygen consumption due to several “hits” on mitochondria. COMET measurements were intraoperatively performed in four patients in the presternal skin region. The patients

underwent cytoreductive surgery of peritoneal metastases of a primary intestinal tumor and during the same operation, patients received hyperthermic intraperitoneal chemotherapy (HIPEC). The chemotherapeutic agent was Mitomycin C and was perfused at 42 °C. During surgery and directly post-operative macrohemodynamic support is given by a generous fluid regime and noradrenaline perfusion. An example of the change in ODR during intraperitoneal chemotherapy is shown in Figure 2A. As shown in Figure 2B, in 3 out of 4 patients COMET measured a reduced ODR independently of mitoPO2 after HIPEC perfusion.

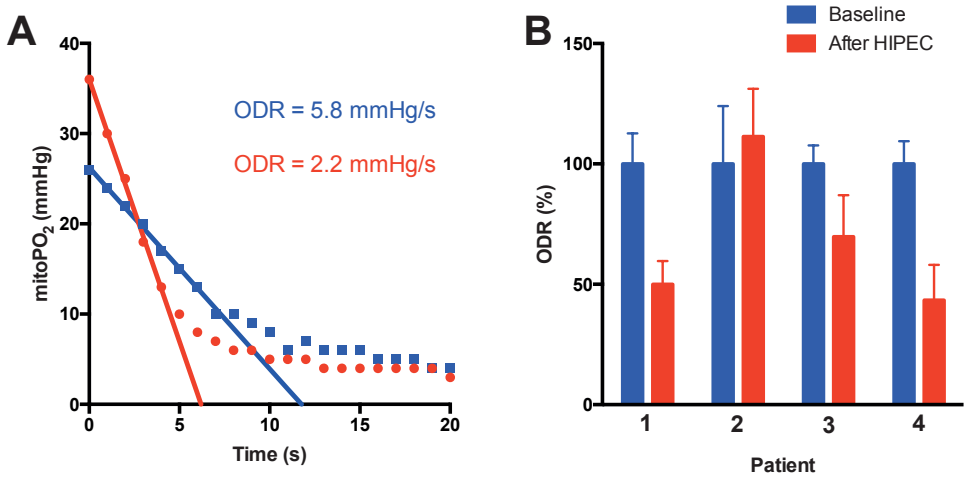

Figure 2 MitoPO2 and ODR during cytoreductive surgery. (A) Typical examples of mitochondrial oxygen tension (mitoPO2) and Oxygen Disappearance Rate (ODR) measurements at start of surgery and after HIPEC perfusion. (B) Normalized ODR of 4 patients. After HIPEC perfusion ODR is shown as percentage compared to baseline, baseline is set to 100% for each individual patient.

Oxygen balance

Mitochondrial oxygen concentration depends on the concentration of hemoglobin and its saturation, the hemoglobin-oxygen disassociation characteristics in microcirculation and microvascular flow. In essence, the oxygen supply needs to meet the demand to prevent hypoxia and cellular adaptation (93). If oxygen supply exceeds demand it will lead to hyperoxia and oxidative stress induces a cellular response (94). Especially in newborn care, negative effects of hyperoxia on lung development have led to a strategy of permissive hypoxia (95,96). Current clinical practice is safeguarding macrohemodynamics and saturation without knowing the exact cellular oxygen concentration (97). Mitochondria are the oxygen consumers, thus maintaining a sufficient oxygen concentration in the

mitochondria is the goal in the blood-tissue oxygen distribution. Therefore, mitoPO$_2$ is the result of the balance between oxygen supply and the cells ability to consume it (as shown in figure 3).

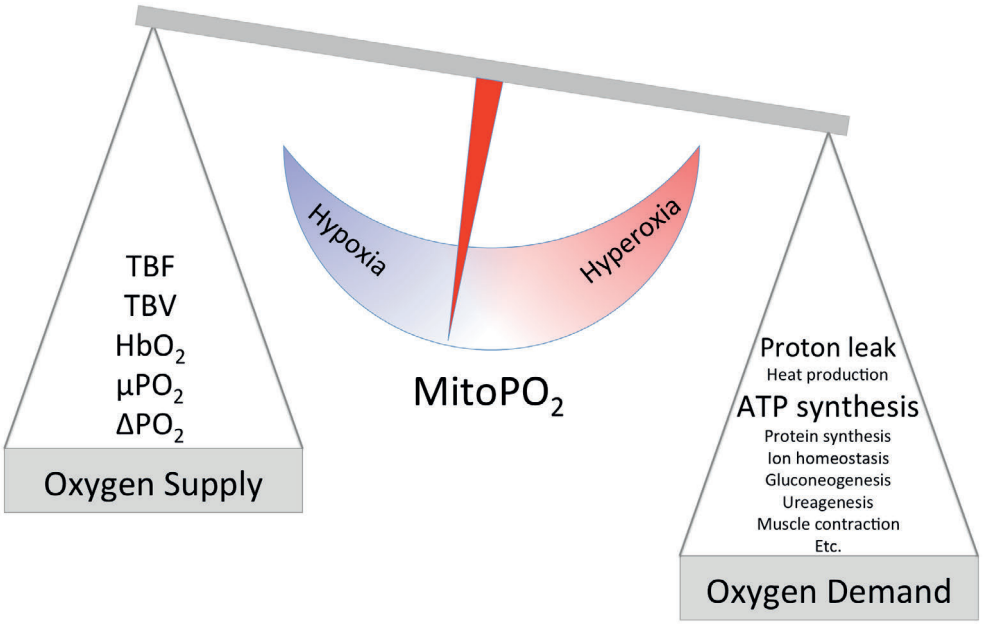

Figure 3. The oxygen balance. Mitochondrial oxygen tension (mitoPO$_2$) is a resultant of mitochondrial oxygen supply and demand. The respiratory chain occupies the demand side, and the biggest part of its oxygen consumption is coupled to synthesis of adenosine triphosphate (ATP). A small part is used for energy dissipation and heat generation by uncoupling via proton leak over the mitochondrial inner membrane (9). Oxygen supply is dependent on tissue blood flow (TBF), tissue blood volume (TBV), local hemoglobin saturation (HbO$_2$), microvascular oxygen tension (μPO$_2$), and factors influencing the oxygen gradient between microvessels and mitochondria (ΔPO$_2$). Reprinted with permission from: Mik (17)

Conclusion

Since a primary goal of caregivers is maintaining supply and demand, mitochondrial oxygen levels and oxygen consumption might be useful parameters for monitoring the effectiveness of therapies in optimizing oxygen supply. Achieving oxygen hemostasis, and thus maintaining cellular oxygen concentration and oxygen metabolism within a normal range may be an ultimate goal. COMET is able to provide insight in the oxygen balance at tissue level, and thus provides important information about aspects of mitochondrial function. Considering the complexity of the pathophysiology of a critically ill patient,

especially the patients with severe sepsis and septic shock, a multilevel approach is needed. In understanding the interplay between macrocirculation, microcirculation and parenchymal cells the mitochondria are key players that should not be overlooked.

Conflict of interest statement

EGM is inventor of the technique for measuring mitochondrial oxygen tension, as used in the COMET measuring system. He is founder and shareholder of Photonics Healthcare B.V., Utrecht, The Netherlands. Photonics Healthcare develops and markets the COMET system. All other authors declare no conflict of interest.

Author contributions

All authors contributed specific parts to the manuscript and read, corrected and approved the whole manuscript. Mark Wefers Bettink contributed with Hits on mitochondria, Cytopathic hypoxia, Ways to measure mitochondria and current insights and COMET. Sesmu Arbous contributed with the part on Hemodynamic coherence. Harold Raat contributed with the part on Metabolic adaptation. Egbert Mik drafted the manuscript and wrote the Abstract, Introduction, Aspects of mitochondrial function and Conclusion.

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Chapter 3

A monitor for Cellular Oxygen METabolism (COMET): monitoring tissue oxygenation at the mitochondrial level

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Abstract

After introduction of the protoporphyrin IX-triplet state lifetime technique as a new method to measure mitochondrial oxygen tension *in vivo*, the development of a clinical monitor was started. This monitor is the "COMET", an acronym for Cellular Oxygen METabolism.

The COMET is a non-invasive electrically powered optical device that allows measurements on the skin. The COMET is easy to transport, due to its lightweight and compact size. After 5-aminolevulinic acid application on the human skin, a biocompatible sensor enables detection of PpIX in the mitochondria. PpIX acts as a mitochondrially located oxygen-sensitive dye. Three measurement types are available in the touchscreen-integrated user interface, 'Single', 'Interval' and 'Dynamic measurement'.

COMET is currently used in several clinical studies in our institution. In this first description of the COMET device we show an incidental finding during neurosurgery. To treat persisting intraoperative hypertension a patient was administered clonidine, but due to rapid administration an initial phase of peripheral vasoconstriction occurred. Microvascular flow and velocity parameters measured with laser-doppler (O2C, LEA Medizintechnik) decreased by 44% and 16% respectively, but not the venous-capillary oxygen saturation. However, mitochondrial oxygen tension in the skin detected by COMET decreased from a steady state of 48 mmHg to 16 mmHg along with the decrease in flow and velocity.

We conclude that COMET is ready for clinical application and we see the future for this bedside monitor on the intensive care, operating theater, and testing of mitochondrial effect of pharmaceuticals.

Keywords: [PpIX-TSLT, COMET, Mitochondrial oxygen tension (mitoPO_2), Diagnostics, Tissue Oxygenation]

1. Introduction

Because of the importance of adequate tissue oxygen supply, many techniques have been developed for measuring oxygen *in vivo* over the last decades[1, 2]. The ultimate goal of measuring oxygen at the level of the mitochondria has recently become reality. We introduced the protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT) for measuring mitochondrial oxygen tension (mitoPO_2) in 2006 [3]. In the mean time, the technique has been proven to be useful in isolated cells, isolated organs and *in vivo* in animal studies [4–7].

PpIX-TSLT is based on the principle of oxygen-dependent quenching of the excited triplet state of protoporphyrin IX (PpIX). Application of the porphyrin precursor 5-aminolevulinic acid (ALA) induces PpIX in the mitochondria where it acts as a mitochondrial located oxygen-sensitive dye. After photo-excitation with a pulse of green light PpIX emits delayed fluorescence of which the lifetime is inversely related to the amount of oxygen. The technique is non-invasive and can be safely used in humans [8].

The ability to measure optically intracellular oxygen is providing the possibility to assess oxygenation at the end of the oxygen cascade [3, 9]. Measurements in the intracellular compartment are complementary to for example hemoglobin-based oxygen measurements. Pulse-oximetry typically measures at the arteriolar side of the microcirculation [10] while near-infrared and visible light spectroscopy are biased toward the venous compartment [11, 12]. Interstitial oxygen measurements with e.g. oxygen electrodes measure close to the cellular compartment, but are cumbersome and tissue destructive [1]. Measuring at the end of the oxygen cascade is important since (pathologic) shunting in the microcirculation or the development of tissue edema can cause cellular hypoxia, which is otherwise not detectable [13].

Besides measuring mitoPO_2 PpIX-TSLT also provides the possibility to get insight in local tissue oxygen consumption at the mitochondrial level [14]. Mitochondrial oxygen consumption (mitoVO_2) can be estimated by measuring the oxygen disappearance rate (ODR) in the measuring volume [15]. Recently we have demonstrated that this enables bedside non-invasive monitoring of an important aspect of mitochondrial function in animal models of critical illness [16, 17].

A clinical device featuring PpIX-TSLT has now been developed and recently entered use in clinical trials in our institution. This monitor is called “COMET”, an acronym for Cellular Oxygen METabolism. The COMET measuring system enables physicians to measure

oxygen tension and oxygen consumption at the subcellular level in the mitochondria. This paper is the first description of this CE-marked device (Photonics Healthcare, Utrecht, The Netherlands). It provides the technical background, the construction of the device, and its use together with two examples of measurements in human skin.

2. Methods

2.1 Background of PpIX-TSLT

Protoporphyrin IX (PpIX) is the final precursor of heme in the heme biosynthetic pathway and is synthesized inside the mitochondria [13]. The conversion of PpIX to heme in the mitochondria is a rate-limiting step. Therefore, administration of the porphyrin precursor 5-aminolevulinic acid (ALA) enhances the mitochondrial PpIX concentration [18]. Administration of ALA does not only enhance PpIX to detectable levels, but it also ensures mitochondrial origin of the delayed fluorescence signal [3, 6, 7].

Delayed fluorescence can be observed after pulsed excitation of PpIX as delayed luminescence with the same spectrum as prompt fluorescence (red light). In contrast to prompt fluorescence delayed fluorescence has a lifetime of tens to hundreds of microseconds [3]. Delayed fluorescence is the result of photon emission due to spontaneous relaxation of the excited triplet state via bi-directional intersystem crossing. Oxygen is a very effective quencher of the excited triplet state. In the process of quenching, energy is transferred to oxygen and PpIX relaxes to the ground state without emission of a photon. This causes the lifetime of the triplet state, and thus the lifetime of the emitted delayed fluorescence, to be oxygen-dependent.

The delayed fluorescence lifetime is inversely proportional to the amount of oxygen according to the Stern-Volmer equation [8, 19] . With the assumption of a homogenous distribution of oxygen this relationship can be used to calculate the mitochondrial oxygen tension:

$$MitoPO_2 = \frac{\frac{1}{\tau} - \frac{1}{\tau_0}}{k_q} \quad (1)$$

Where τ is the measured delayed fluorescence lifetime, τ_0 is the delayed fluorescence lifetime in the absence of oxygen (i.e. the lifetime of spontaneous relaxation), and k_q is the quenching constant.

2.2 Signal analysis

Oxygen however is heterogeneously distributed in tissues *in vivo*. Previous studies have shown that this also applies for mitoPO$_2$ [6, 7, 13]. Delayed fluorescence from a heterogeneous system does not decay mono-exponentially, but the signal contains a lifetime distribution. Fitting equation 1 to a distribution of lifetimes generally leads to an underestimation of the mean PO$_2$ in the measuring volume [20]. A much better estimation of the mean PO$_2$ can be found by alternatively fitting a distribution of quencher concentration to the delayed fluorescence signal. The fitting function for a simple rectangular distribution with a mean mitoPO$_2$ Q$_m$ and a mitoPO$_2$ range from Q$_m$-δ till Q$_m$+ δ is [21]:

$$Y_R(t) = \exp\left(-\left(\frac{1}{\tau_0} + k_q\langle mitoPO_2\rangle\right)t\right)\frac{\sinh(k_q\delta t)}{k_q\delta t} \qquad (2)$$

where Y$_R$(t) is the normalized delayed fluorescence data, <mitoPO$_2$> is the mean mitoPO$_2$ within the sample volume and t is the factor time.

Fitting of equation 2 is fast and very robust when applied to weak delayed fluorescence signals and noisy real world signals. In a previous analysis we have shown that fitting equation 2 allows for reliable retrieval of mitoPO$_2$ values from data with signal-to-noise ratios (SNR) as low as 10 [5]. For time-domain delayed luminescence measurements SNR is defined as the maximum amplitude of the delayed fluorescence divided by the maximum amplitude of the noise. Generally a SNR above 20 is well achievable and the noise-induced error in the measurement remains below 2%.

For analysis of the delayed fluorescence signals COMET uses equation 2 to calculate mean mitoPO$_2$ in the measuring volume under the probe. The absolute value for mitoPO$_2$ is directly displayed on the screen without further processing. COMET also evaluates the signal quality, which is calculated from the SNR value; an increase of 1 in SNR is approximately 1% in signal quality up till a SNR of 50. Beyond a SNR 50 the increase in signal quality percentage will flatten out. As long as SNR is within an acceptable range, a SNR greater than 5, COMET will show a percentage and a calculated mitoPO$_2$. If COMET cannot detect a signal, or SNR is too low, less than or equal to 5, the used version of the firmware makes COMET to display “no signal found” and to provide the unrealistic value of “999”.

2.3 Monitor description

The COMET is a medical device and class IIa classified according to the Medical Device Directive 93/42/EEC. The legal manufacturer is Photonics Healthcare B.V., Utrecht, The Netherlands. It weighs 10 kg and sizes 22 x 33 x 29 cm without cradle and port cover on the side. The COMET measurement system exists of two components shown in figure 1. The first component is the monitor which includes the multi-touch screen integrated user interface, light source, detection system and processing units. The second component is the COMET Skin Sensor developed for use on the human skin.

Fig.1 COMET monitor and skin sensor.

2.3.1 Hardware

The COMET is an electrically powered system (rated power consumption of 250 W). The light source and the detection system are the two core components. A 515 nm pulsed laser, pulse duration 60ns, with a 10Hz repetition rate illuminates the intra cellular accumulated PpIX. The fluorescent signal is projected on a gated red-sensitive

photomultiplier tube. Users can interact via a multi-touch 12" TFT-LCD screen. Apart from the main switch to turn on the device, the COMET has no physical buttons. If a USB storage device is inserted in the USB-port on the rear panel the data is exported in a comma separated file format for further processing in programs like MS Excel. The COMET can be used on a flat surface or be mounted on a trolley or arm through a VESA 75/100 compatible adapter plate.

2.3.2 Software

Lifetimes of the raw data are calculated on an embedded control board. The embedded calculation software is written in C code to simplify the development process as per IEC 62304 as required for certification. The user interface (UI) is running on a separate Linux based operating system to enhance device usability experience.

There are three different types of measurement to distinguish, shown in table 1.

Table 1 The COMET different measurement types.

Single measurement	One measurement per activation of the touchscreen non physical button.
Interval measurement	The COMET will measure in a set interval: At the start of the interval a measurement is done, and the interval time can be chosen (60 min, 20 min, 5 min, 1 min)
Dynamic measurement	The COMET can conduct a series of up to 120 measurements, one measurement per second.

2.4 Location of the measurement

The COMET measures oxygen tension in mitochondria by measuring the triplet-state lifetime of PpIX. Under normal (non-sensitized) conditions PpIX is present in very low concentrations in the human skin and not detectable with the COMET. This can be overcome by the exogenous administration of ALA that leads to higher concentrations of PpIX in the mitochondria.

ALA synthase is the first and the rate-limiting enzyme of the porphyrin synthetic pathway. Under normal conditions the level of heme synthesis and the intracellular concentration of PpIX are mainly regulated by heme control of the ALA synthase activity. As a small molecule, ALA penetrates the stratum corneum [22]. Exogenously provided ALA bypasses the negative feedback controls in the heme biosynthetic pathway and leads to overproduction of PpIX [23].

The COMET can measure in healthy skin as well as in skin lesions. After ALA application the measurement area needs to be covered, to avoid consumption of PpIX by light. A priming time for ALA, typically 4 hours or more, is needed to synthesize a suitable concentration of PpIX to enable measurements of mitoPO_2 and oxygen disappearance rate.

After topical administration on healthy skin, PpIX is synthesized in the epidermis but not (significantly) in the dermis [24]. While ALA penetrates into the dermis the heme-synthesis pathway in most dermis cells is inactive. The conversion of ALA to PpIX requires energy and an intact heme cycle, thus PpIX is not synthesized in the metabolically inactive cells of the stratum corneum. In healthy skin this limits the intradermal measurement location and signal origin of the COMET to the epidermis with a thickness of about 0,1mm [25].

The recommended measurement location is the skin of the sternum seen in figure 2. This provides a central measurement location less influenced by temperature changes, movement and peripheral vasoconstriction [26].

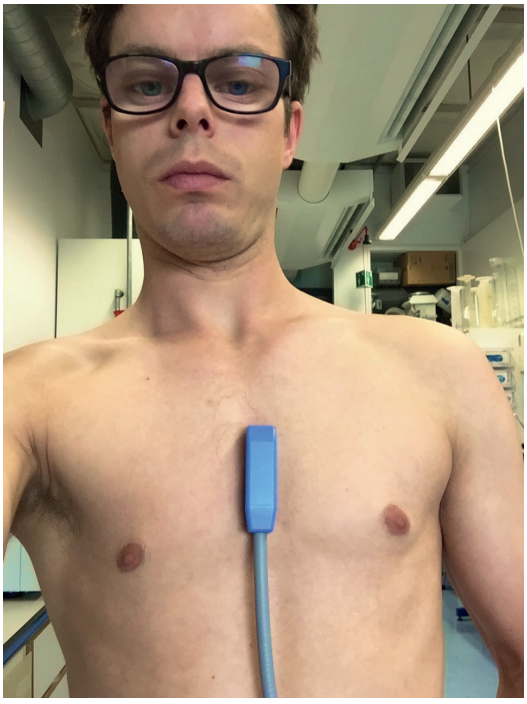

Fig .2 COMET skin sensor position on the sternum

2.5 Skin sensor

The biocompatible housing (70x20x20mm) of the Skin Sensor, shown in figure 3, holds two optical fibers; the excitation and the detection fiber. A flexible metal tube protects the vulnerable optical fibers against external mechanical forces. The optical design of the sensor can collect light at approximately a right angle to the sensor cable. The light emitted by the sensor is divergent and safe for eyesight at any distance.

Ambient light entering the detection path might overload or even damage the photomultiplier tube. For protection, a photodiode in the Skin Sensor determines the ambient light before each measurement. Sensor temperature, used as an approximation of skin temperature, is measured with an electrical resistive sensor.

Fig. 3 Detailed view of skin sensor

2.7 Oxygen-consumption measurement

The COMET provides the opportunity for measurements in dynamic situations by taking a series of 120 samples of the mitoPO_2 acquired at 1Hz. This can be used to determine the oxygen disappearance rate (ODR) and reperfusion. Typically, mitochondrial oxygen availability is measured for 10-20 seconds in an undisturbed and stable situation. Subsequently light pressure is applied with a hand onto the sensor, to give occlusion of the microcirculation and stop local blood flow in the measurement volume, for about 45 seconds. After these 45 seconds the pressure is released and restoration of microcirculatory blood flow and mitochondrial re-oxygenation will appear.

Previously, we have described the fundamental principles behind the technology and have provided a working implementation of the technique for mitoVO_2 measurements in vivo [14]. In summary, the ODR is generally dependent on two factors; oxygen consumption

(VO_2(t)) and Diffusive Oxygen Influx into the measurement volume (DOI(t)). The method to calculate ODR from the mitoPO_2 kinetics is:

$$ODR = dPO_2/dt = -VO_2(t) + DOI(t)$$

The VO_2(t) is oxygen-dependent and, according to Michaelis-Menten kinetics, can be described as:

$$VO_2 = (V_{max} \cdot PO_2(t))/(P_{50} + PO_2(t))$$

Where V_{max} is the not supply-dependent maximal tissue oxygen consumption and P_{50} is the PO_2 at which cellular oxygen consumption is reduced to ½ V_{max}. PO_2(t) denotes the PO_2 in the measurement volume at time point t.

2.8 In human measurements

Using COMET a dynamic measurement was performed on a healthy volunteer. Preceding the measurements an ALA plaster 4 cm^2 (2mg 5-amino-4oxopentacid / cm^2) was applied for approximately 10 hours (overnight) onto the skin of the sternum. A baseline of 20 seconds was measured before the microcirculation was occluded. The microcirculation occlusion was accomplished by application of external pressure by hand on the Skin Sensor.

Secondly, we report an incidental finding we made during an ongoing feasibility study of the COMET. The study is performed in accordance with the declaration of Helsinki and patients are consented with a protocol approved by local ethics committee METC (CCMO number NL51937.078.15). This study is set up to determine the applicability, stability, and reproducibility of the COMET measurement over a longer period of time during neurosurgery. The shown incidental finding is an observation that occurred during non-protocolled administration of the central alpha-receptor agonist clonidine.

MitoPO_2 was measured intraoperatively, simultaneously to tissue oxygenation saturation and perfusion parameters (O2C, oxygen to see version 2424, Lea Medizintechnik GmbH, Germany). The O2C measures three parameters: The local capillary venous saturation (SO_2), the local velocity of blood given in velocity units (VU) and the local micro vascular blood flow given in flow units (FU). Both the COMET Skin Sensor and the O2C probe (LFX-43) were positioned on the sternum next to each other.

3. Results

3.1 Oxygen-consumption measurement

A typical example of an oxygen-consumption measurement on the healthy volunteer, is shown in figure 4. Mean mitoPO$_2$ (t_0-$_{19}$) gave a baseline mitoPO$_2$ of 22.7 ± 2.1 mmHg (mean ± SD). After 20 seconds direct pressure with the probe was given to occlude microvascular blood flow in the skin. The available oxygen was consumed and resulted in an oxygen disappearance rate of 6.3 mmHg.s^{-1}. When the pressure was released and direct oxygen recovery up to 60-70 mmHg in mitoPO$_2$ was seen. At 120 seconds the mitoPO$_2$ returned to baseline values.

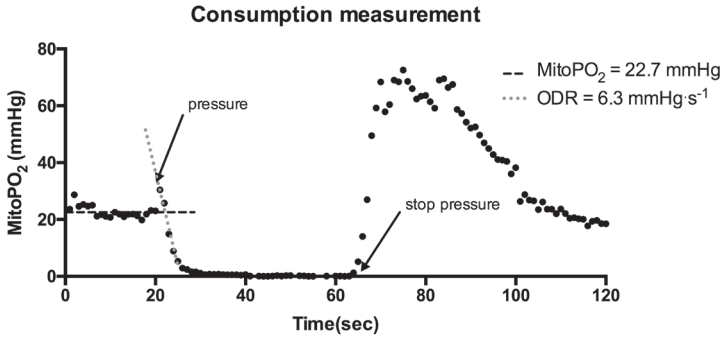

Fig.4 A typical dynamic measurement of mitochondrial partial oxygen pressure (mitoPO$_2$) by COMET measurement system. A sample of 120 seconds is shown. In the first 20 seconds the baseline was determined, afterwards light pressure was applied on the sensor to stop microcirculation and the oxygen disappearance rate (ODR) was measured. At 60 seconds pressure was released.

3.2 Incidental finding during ongoing clinical study

In one of the measured patients during an ongoing feasibility study in neurosurgery patients, clonidine was given intravenously due to persistent hypertension. Clonidine is a central inhibitor of noradrenergic neurotransmitter transmission but also a peripheral a1-agonist. Given in a short period of time clonidine leads to initial peripheral vasoconstriction, followed by a slow onset of vasodilatation. In this particular case 150 microgram of clonidine was given as a bolus application. A direct onset effect of vasoconstriction on flow and velocity but not on capillary venous oxygen saturation (SO_2) was seen as measured by O2C figure 5. Flow decreased by 44% and velocity by 16%. Although SO_2 did not change, a transient drop in mitoPO$_2$ was measured with the COMET. MitoPO$_2$ dropped from a steady state of 48 mmHg to 16 mmHg. After the fast clonidine

administration the restoration of blood flow and velocity, mitoPO$_2$ returned to baseline in approximately 15 minutes.

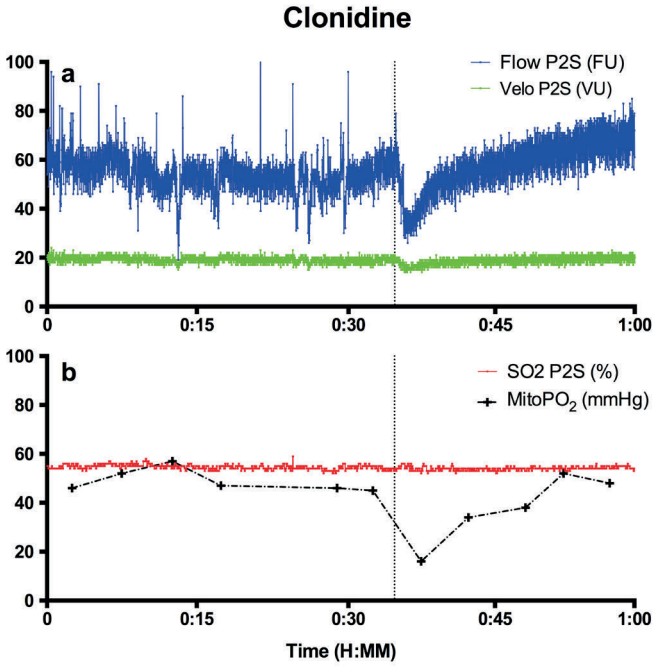

Fig.5 A Flow in arbitrary flow units (FU) and arbitrary velocity in velocity units (VU) of microcirculation as measured with probe 2 (P2S) O2C. **B** Capillary venous saturation (SO2) as measured by O2C and mitochondrial partial oxygen pressure (mitoPO$_2$) as measured by the COMET.

4. Discussion

The COMET measurement system is the successor of the first clinical prototype previously described in this journal [17]. This new clinical monitor is safe to be used, easy to transport and applicable for *in vivo* measurement of mitochondrial oxygen tension and consumption in the human skin. The sternal skin is an easy accessible and non-invasive measurement location. The clinical significance of measuring in the skin arises from the fact that, like the gut, the skin can be regarded as a canary of the body [27]. The idea is that cutaneous mitochondrial PO$_2$ changes foretell changes in other vital organs and systemic parameters. Indeed, in an animal model mitoPO$_2$ appeared an earlier indicator of approaching the limit of physiological compensation during hemodilution than e.g. venous saturation and lactate [27, 28]. The “canary” function of human skin in relation

to organ function still needs further investigation but important is that also changes in cutaneous mitochondrial oxygen consumption correlate with ODR changes in other organs and tissues [17]. The ODR from the dynamic measurement 6.3 $mmHg.s^{-1}$ in this example corresponds with previous data from healthy volunteers 5.8 ± 2.3 $mmHg.s^{-1}$ [29] .

In this paper we describe the case of clonidine given to a patient within a short period of time. This incidental finding occurred during an ongoing neurosurgery feasibility study. This patient group was chosen because in general we aim at hemodynamic stability during surgery over a longer time period. Therefore the incidental finding of an abrupt drop in cutaneous mitoPO_2 after a bolus clonidine came clearly forward. In line with clinical observations the effects of clonidine administration resulted in initial vasoconstriction and subsequent vasodilatation after a couple of minutes. Using online monitoring we observed a direct decline in microvascular blood flow and velocity, followed by an increase in flow and velocity as measured by the O2C. With the change of flow and velocity a decrease in the oxygen supply to the tissue is expected. In consequence a decrease in mitoPO_2 of 30mmHg is observed as measured by the COMET.

However, interestingly, the capillary-venous oxygen saturation measured by the O2C did not show any decrease in the minutes following clonidine administration. Two main phenomena could explain the unchanged capillary-venous oxygen saturation. First, the SO_2 is measured with the absorbance of visible light; the velocity and flow are measured with the hemoglobin laser doppler frequency shift. The different wavelengths of light, giving a different tissue penetration and therefore measurement compartment [30, 31], used in these techniques may explain why a difference in flow but not of SO_2 after clonidine administration could be observed. A second explanation could be a total stop flow of some capillaries. The part of the capillary tree without flow does not contribute to venous-capillary saturation. Thus, the oxygen extraction in the measurement volume stays the same. For the COMET measurements heterogeneity of the oxygen content in the measurement volume could be demonstrated [8]. Therefore a heterogeneous bimodal distribution could explain the decrease in flow and a constant oxygen capillary venous saturation. The reader should keep in mind that for practical reasons the O2C and COMET measurements were performed in close proximity of each other, but not in exactly the same area of the skin. However, we do think that for a valid comparison of the measurements the fact that SO_2 and mitoPO_2 were measured at the same depth in skin is of more importance. Based on our findings it is clear that measuring oxygen availability directly at cellular level provides complementary data and new insight.

While COMET is the first clinical device for measuring mitochondrial oxygen and oxygen consumption, the used technology has some limitations. Currently the typical application time of the ALA on the skin is 4 hours. This makes the measurement technique not yet applicable in acute situations. Furthermore, the combination of topical ALA administration and the green excitation light cause a very shallow measurement depth. While this does enable the oxygen consumption measurements, the oxygen measurements become more sensitive to tissue heterogeneity and background light.

Till now the feasibility of measuring mitoPO$_2$ and mitoVO$_2$ with PpIX-TSLT was tested in healthy volunteers [29] and is currently further evaluated with COMET in the perioperative setting. However, the original main development idea of COMET was the *in vivo* determination of aspects of mitochondrial (dys)function in critical illness. Indeed, in the laboratory setting the effectiveness of this technique in determining mitochondrial function under septic circumstances could be demonstrated [16].

Furthermore, mitochondrial oxygen measurements could potentially provide a new physiological transfusion trigger for decision-making in transfusion medicine. In a very recent animal study we have shown that mitoPO$_2$ can be used as an early detector of reaching the individual limit of hemodilution before changes in systemic oxygen consumption and lactate levels occur [27]. If this concept can be translated into the anemic human situation, it is indeed potentially an individual physiological parameter to guide blood transfusions. The technique used in COMET is not limited to measurements in skin, since ALA can be administered systemically [32, 33]. Therefore, endoscopic or intraoperative measurement of mitoPO$_2$ is technically feasible but such attempts should always take into account extensive safety considerations related to potential photodynamic toxicity.

4. Conclusion

This report provides a description of the novel COMET measurement system. The enhanced protoporphyrin IX concentration in the skin is used as endogenous oxygen-sensitive probe. The method gives the possibility to measure cellular oxygen availability and the oxygen disappearance rate at the bedside on a mitochondrial level. In the future the COMET could play a role in clinical practice to assess tissue viability, to manage oxygen transport, and to recognize and possibly to treat mitochondrial inhibition in critically ill patients. Furthermore it potentially can be used as an individual blood transfusion trigger and may enable testing mitochondrial effects of pharmaceutical substances research.

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Conflicts of interest statement

Dr. E.G. Mik and F. Michael Münker are founders and shareholders of Photonics Healthcare, a company aimed at making the delayed fluorescence lifetime technology available to a broad public. Photonics Healthcare B.V. holds the exclusive licenses to several patents regarding this technology, filed and owned by the Academic Medical Center in Amsterdam and the Erasmus Medical Center in Rotterdam, The Netherlands.

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Chapter 4

Mitochondrial oxygen monitoring with COMET: verification of calibration in man and comparison with vascular occlusion tests in healthy volunteers

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Abstract

Mitochondria are the primary consumers of oxygen and therefore an important location for oxygen availability and consumption measurement. A technique has been developed for mitochondrial oxygen tension ($mitoPO_2$) measurement, incorporated in the COMET. In contrast to most textbooks, relatively high average $mitoPO_2$ values have been reported. The first aim of this study was to verify the validity of the COMET calibration for $mitoPO_2$ measurements in human skin. The second aim was to compare the dynamics of $mitoPO_2$ to several other techniques assessing tissue oxygenation.

Firstly, we performed a two-point calibration. Mitochondrial oxygen depletion was achieved with vascular occlusion. A high $mitoPO_2$ was reached by local application of cyanide. $MitoPO_2$ was compared to the arterial oxygen partial pressure (PaO_2). Secondly, for deoxygenation kinetics we compared COMET variables with the LEA O2C, SenTec OxiVenT™ and Medtronic INVOS™ parameters during a vascular occlusion test.

Twenty healthy volunteers were recruited and resulted in 18 datasets (2 times 9 subjects). The lowest measured $mitoPO_2$ value per subject had a median [IQR] of 3.0 [1.0 – 4.0] mmHg, n=9 . After cyanide application the $mitoPO_2$ was 94.1 mmHg [87.2 – 110.9] and did not differ significantly (n=9, p=0.5) from the PaO_2 of 101.0 [98.0 – 106.0] mmHg. In contrast to O2C, OxiVenT™ and INVOS parameters, $mitoPO_2$ declined within seconds with pressure on the probe. The kinetics from this decline are used to mitochondrial oxygen consumption ($mitoVO_2$).

This study validates the calibration of the COMET device in humans. For $mitoVO_2$ measurements not only blood flow cessation but application of local pressure is of great importance to clear the measurement site of oxygen-carrying erythrocytes.

1. Introduction

Mitochondria are small intracellular organelles that generate energy for the cells in the form of adenosine triphosphate (ATP). Oxygen is of critical importance for efficient ATP generation through the process of oxidative phosphorylation, also called mitochondrial respiration. This function makes mitochondria the primary consumers of oxygen in the body and therefore the most desired location for measuring oxygen availability and consumption.

An optical noninvasive technique has been developed for measuring mitochondrial oxygen tension (mitoPO$_2$). MitoPO$_2$ is determined with the protoporphyrin IX-Triplet State Lifetime Technique (PpIX-TSLT) by measuring the oxygen-dependent delayed fluorescence lifetime of 5-aminolevulinic acid (ALA)-induced PpIX [1–3]. This measurement technique is incorporated in a medical device called the Cellular Oxygen METabolism monitor (COMET)[4].

Previous studies that used the protoporphyrin IX lifetime technique for cutaneous mitoPO$_2$ measurements in humans reported some remarkable results. Most importantly, relatively high average mitoPO$_2$ values of around 44 mmHg (5.9 kPa) [5] and 66 mmHg (8.8 kPa) [6] have been reported. In contrast, most textbooks mention normal values of mitochondrial oxygen tension as low as 7.5 mmHg (1 kPa) or less [7]. The calibration constants used in the COMET have been determined in animal studies [8]. A direct calibration in man has been lacking to preclude the high PO$_2$ values being a result of improper calibration. The first aim of this study was therefore to verify the calibration of COMET in human skin.

No other clinical device is able to measure oxygenation at the mitochondrial level at the bedside. A direct comparison with other measurement techniques is thus unreliable because every tissue compartment, from intravascular to intracellular, has a different oxygen tension, leading to oxygen gradients. Due to the lack of a gold standard we aimed at using the same approach as used for in vivo calibration in animals, i.e. to use a combination of blocking oxygen supply by microvascular occlusion and blocking mitochondrial respiration by cyanide cream [8]. This provides a two-point calibration with a minimal mitoPO$_2$ value during microvascular occlusion and a known mitoPO$_2$ value after blockage of mitochondrial oxygen consumption.

Next to mitoPO$_2$ measurements, the COMET system can be used to assess the parameters mitoVO$_2$ (a measure for oxygen consumption) and mitoDO$_2$ (as a measure for oxygen

delivery) [3, 6]. Several methods have been developed over the years for measuring tissue oxygen consumption non-invasively. Most of these methods use hemoglobin-based measurement techniques, measuring a vascular or microvascular hemoglobin oxygen saturation in combination with a vascular occlusion test [9]. Typically, the mitoVO$_2$ measurements with COMET show much faster deoxygenation kinetics than those other approaches.[10] Therefore, the second aim of this study is to compare COMET variables to spectroscopic and transcutaneous techniques during vascular occlusion testing.

In short, in this study we compare mitoPO$_2$ with an arterial blood gas to verify the validity of the COMET calibration for mitoPO$_2$ measurements in human skin and compared the dynamics of mitoPO$_2$ to several other techniques for assessing tissue oxygenation in a series of healthy volunteers.

2. Methods

The study was approved by the local medical ethical committee and registered on www.toetstingonline.nl [NL61767.078.17]. The study complies with the Helsinki declaration on research ethics. Healthy volunteers were recruited at Erasmus Medical Center Rotterdam, the Netherlands. Informed consent was obtained prior to participant inclusion. Inclusion criteria were: subjects between 18 and 50 years of age and ASA-1-2. Exclusion criteria were: mental disability, presence of mitochondrial disease, diabetes, anemia, hemoglobinopathy, mild to severe COPD, porphyria and/or use of anti-coagulant medication.

2.1 Measuring mitochondrial oxygen tension

The COMET (Photonics Healthcare BV, Utrecht, The Netherlands) was used for mitoPO$_2$ and mitoVO$_2$ measurements. COMET uses the protoporphyrin IX triplet state lifetime technique (PpIX-TSLT) to measure oxygen availability. It provides quantitative measures, does not affect the measured tissue, and does not need recalibration before use [1]. For the extensive description of COMET internal components and the implemented algorithm, we refer to previous work [4]. We have described the fundamental principles behind the technology and have provided a working implementation of the technique as well as a method for calculating mitoVO$_2$ from the mitoPO$_2$ kinetics [11].

Before mitoPO$_2$ measurements can be performed 5-Aminolevulinic acid (ALA) has to be applied to the skin to induce sufficient mitochondrial PpIX for detection of delayed

fluorescence [1, 12]. To this end we cutaneously applied a 4 cm^2 plaster, containing 8mg ALA (Photonamic, Hamburg, Germany). Six to eight hours previous to the measurements the ALA-plaster was applied to the lower arm. The COMET Skin Sensor was fixated onto the skin using a double-sided adhesive transparent plaster without optical interference (LEA Medizintechnik GmbH, Giessen, Germany).

2.2 Verification of COMET calibration

Since no gold standard exists to which COMET can be compared, verification of COMET calibration had to rely on creating predictable mitochondrial oxygen levels. We chose a two-point verification aiming at approximating zero oxygen conditions and arterial oxygen tension. In earlier experiments in cells and animals the oxygen tension was decreased by flushing or breathing nitrogen to wash out all available oxygen.

In healthy human volunteers tissue-deoxygenation with nitrogen to a near-zero level is not a safe and viable option. A method that is applicable in humans is temporal arterial occlusion of a limb in combination with local pressure on the measuring probe. Arterial and microvascular occlusion inhibits blood flow and thus the oxygen supply to the measurement site. Ongoing cellular oxygen consumption will decrease local mitochondrial oxygen tension to very low values, approximating the desired zero oxygen conditions.

In addition to measurements near zero oxygen conditions we applied a method to compare mitoPO_2 to arterial oxygen tension in a blood gas sample, in order to create a second calibration point at a higher PO_2 level. A known high mitochondrial oxygen tension can be achieved by abolishing the oxygen gradient between arterial blood and the tissue cells. After cessation of mitochondrial oxygen consumption diffusion equilibrates the mitochondrial and arterial oxygen tension. Mitochondrial respiration can be temporarily inhibited by locally applying cyanide [13–15], which has previously been demonstrated in cells and animals [8]. In the transient absence of mitochondrial oxygen metabolism, the measured mitoPO_2 can be compared to the oxygen partial pressure measured in an arterial blood gas (ABG) sample [13–15].

To diminish the influence of external factors like temperature and atmospheric oxygen a gas-sealed incubator was used to control internal air temperature and oxygen concentration, as shown in Figure 1a. During the measurements the subject's arm was inserted into the incubator, which was set to an internal temperature of 37 degrees

Celsius. The oxygen concentration within the incubator was measured with a Fibox 4 trace (PreSense Precision Sensing GmbH, Regensburg, Germany). Prior to the mitoPO$_2$ measurements the arterial blood pressure was taken.

The first mitoPO$_2$ measurement was done in the incubator at a low surrounding PO$_2$ achieved by filling the incubator with nitrogen gas (PO$_2$ <5 mmHg), and with the blood pressure cuff pressurized to 50 mmHg above systolic pressure. After verification of the cessation of blood flow with an O2C laser-doppler monitor with an LFX-43 probe (oxygen to see version 2424, Lea Medizintechnik GmbH, Germany), local pressure was applied with the measurement probe of the COMET to empty and occlude the microvessels in the measured tissue in order to perform a dynamic measurement showing the decreasing mitoPO$_2$ (120 measurements at 1 Hz). With the combination of flow cessation with the pressure cuff, local pressure on the sensor, and mitochondrial oxygen consumption the minimal mitoPO$_2$ was determined in the arm during occlusion. The lowest measured mitoPO$_2$ during this measuring sequence was taken as lowest measurable mitoPO$_2$ per subject.

An arterial blood sample was taken from the radial artery to determine arterial oxygen tension (PaO$_2$) with a blood gas analyzer (ABL 800 Flex, Radiometer, Brønshøj, Denmark). Nitrogen gas was mixed with room air to set the oxygen concentration in the incubator to a level equal to the arterial PaO$_2$ (range ±5 mmHg).

In this study, topical application of cyanide was used to equalize mitoPO$_2$ to PaO$_2$ by blocking oxygen consumption at the skin measurement location. Cyanide ions (CN) bind with high affinity to the mitochondrial cytochrome c oxidase, blocking its activity. As a result, electron transport in the enzyme chain of oxidative phosphorylation and subsequently mitochondrial ATP production and mitochondrial oxygen metabolism are inhibited [16]. The applied cyanide cream was locally produced and contained a concentration of 1% potassium cyanide (Sigma-Aldrich, St. Louis, Missouri) mixed with hydrophilic cremor Lanette (Lanette cream I FNA, Bipharma, Weesp, The Netherlands). After 1 minute the cream was removed and the lower arm was placed in the air mixture equal to the sampled PaO$_2$, after which the mitoPO$_2$ was measured.

To test blockage of mitochondrial respiration after topical application of cyanide the absence of oxygen consumption was checked. After 20 measurements in a sequence of 120 measurements at 1 Hz pressure was applied to the COMET skin sensor. Without cyanide a decrease in mitoPO$_2$ within seconds was seen, as illustrated in figure 5a. While the mitochondrial respiration was blocked the mitoPO$_2$ remained constant as illustrated

in figure. 5c. Fifteen minutes after cyanide application the mitoVO$_2$ was measured in the skin to assess recovery of mitochondrial respiration (figure 5d).

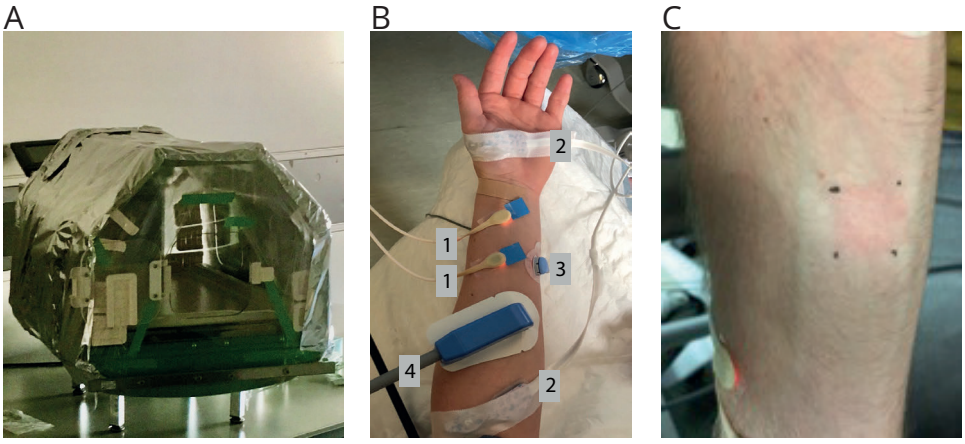


Figure 1: A) *The incubator, sealed to prevent nitrogen gas leakage and provide a controlled air temperature of 37^{0}C.* ***B)*** *Probe position of 1. O2C* LFX-43 probe*, 2. INVOS, 3. SenTec OxiVenT, and 4. COMET Skin Sensor on the lower arm.* ***C)*** *The ALA application side after cyanide application. A temporary hyperemia phase was seen as a red square on the arm.*

2.3 Comparison during vascular occlusion testing

To compare the behavior of mitoVO$_2$ of COMET to other oxygen metabolism-related measurements, a variety of clinical bedside monitoring devices with a temporal resolution of seconds were used. The following devices were included; 1) Oxygen To See with the LFX-43 probe (O2C version 2424, Lea Medizintechnik GmbH, Germany), which combines direct (infra)red light spectroscopy with laser doppler. It measures local capillary venous saturation (SO_2), and local microvascular blood flow is provided in flow units (FU). 2) Near-infrared spectroscopy (INVOS), which measures the tissue saturation and 3) a SenTec Digital Monitoring System with an OxiVenT™ Sensor (SenTec AG, Therwil, Switzerland) which transcutaneously measures blood gases and provides tcPCO$_2$ values. The location of the different probes on the arm can be seen in Figure 1b.

We measured during and after an arterial occlusion test of the arm. Arterial occlusion was achieved by insufflation of a cuff to at least 50 mmHg above the systolic blood pressure. The absence of skin blood flow was confirmed with laser-doppler blood flow measurement with the O2C. During 2 minutes the COMET monitor measured mitoPO$_2$

with a frequency of 1 Hz. The O2C, INVOS, and SenTec OxiVenT all collected data during arterial occlusion to determine the oxygen level and consumption, as well as carbon dioxide (CO_2) accumulation in the measurement volume. In this setup no pressure was applied to the COMET skin sensor in order to be able to compare the deoxygenation rates of the different measurements.

The mitoVO_2 measurement was done with the following procedure; after a stationary measurement of 10 seconds direct pressure on the COMET Skin Sensor probe was applied. This halted the microcirculation, and with it oxygen delivery to the measured tissue volume. The mitoVO_2 was measured directly after local occlusion of the oxygen supply by a linear fit of mitoPO_2. This simple procedure created reproducible stop-flow conditions and induced measurable oxygen consumption rates, consequential to a cessation of microvascular oxygen supply and ongoing cellular oxygen consumption. MitoPO_2 was measured before, during and after application of pressure at an interval of 1 Hz.

2.3 Statistical analysis and software

Software version v.016.5b.184 of COMET was used, during the cyanide measurements, the adjusted timing software was used. Statistical analysis and visualization were done with R version 3.4.2 [17] and GraphPad Prism 6. MitoPO_2 and Arterial Blood Gas were compared using a two sided Wilcoxon-Mann-Whitney U-test. Significance was determined by p-values <0.05. Values are given as median and interquartile ranges or stated otherwise.

The average of the last 3 MitoPO_2 values measured before pressure was applied were used as a baseline. Linear slope comparison (as measure for the deoxygenation rate, or oxygen disappearance rate) between COMET, INVOS, SenTec OxiVenT was performed with GraphPad Prism linear regression model from the descending part of the data. MitoVO_2 linear fit was done with LabVIEW (Version 13, National Instruments, Austin, TX, USA) with the first 4 samples after pressure had been applied by the sensor to compare mitoVO_2 with previous published results [10]. To visually compare the oxygenation decline rate between the devices the data is transformed into z-score (datapoint – mean)/ standard deviation.

3. Results

A total of 20 healthy volunteers were recruited and provided informed consent. Of the 20 volunteers 10 underwent the entire study protocol. One subject dropped out due to beta-thalassemia that was missed during the inclusion procedure. The first 10 inclusions (session 1) resulted therefore in 9 complete datasets. Analysis of this first dataset showed that cyanide application led to unmeasurable delayed fluorescence in all but 1 subject. This unforeseen result was analyzed in cooperation with the manufacturer of the COMET device. It appeared to be caused by the relatively long photomultiplier gate duration in comparison to the very short delayed fluorescence lifetimes after cyanide application, illustrated in Figure 2.

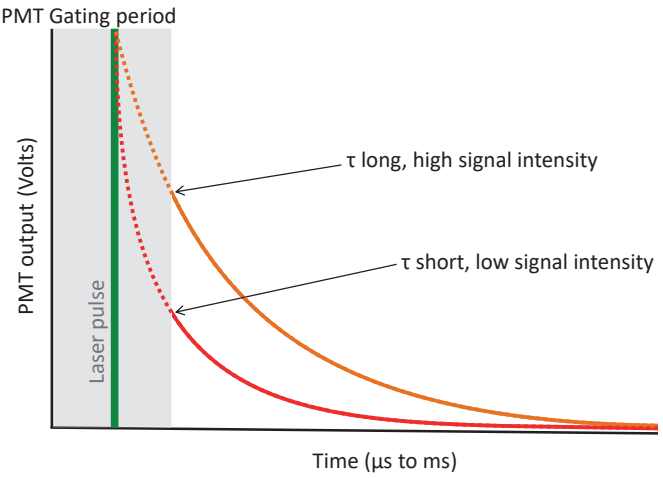
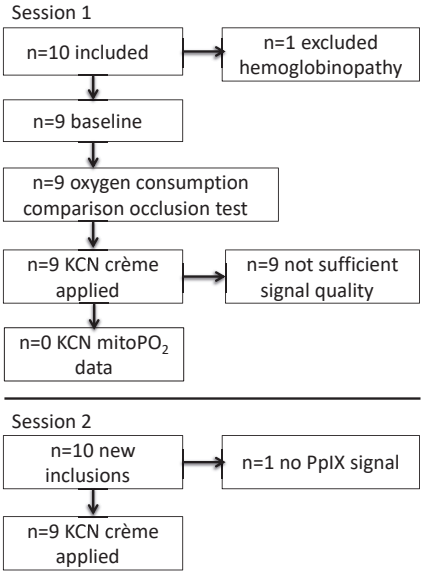


***Figure 2**: This illustration shows the decrease in signal intensity measured by the photomultiplier (PMT) with short lifetimes (several* µs). If the lifetime is short the *oxygen tension is high. The laser pulse (green) is followed by a PMT gating period (gray) to protect the PMT from prompt fluorescence. At the PMT most of the short lifetime signal intensity (red) will be lost. In low oxygen tension and thus a long lifetime (orange) the signal intensity will hardly be influenced by the PMT gating period.*

A temporary change in the firmware of the COMET, kindly supported by the manufacturer, was suggested and used to overcome this problem. Therefore, a second series of measurements (session 2) with the cyanide cream was performed in 10 subjects. One subject had insufficient signal quality after the baseline measurement. This resulted in 9 datasets of new volunteers in the second session. A flow diagram of the inclusions is shown in Figure 3.

A)



B)

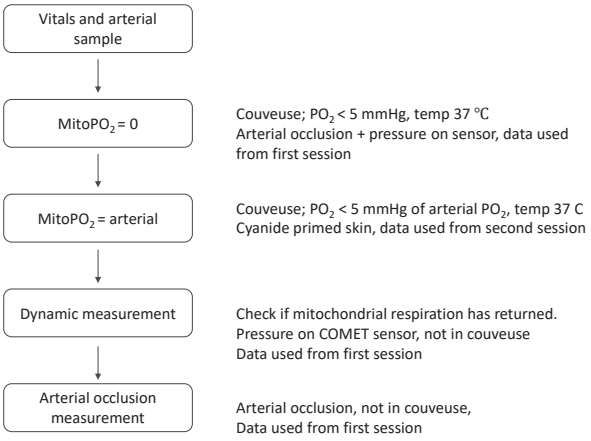


***Figure 3**: A)Diagram of study subject flow, B) Diagram of methods timeline*

Demographic characteristics of the 18 analyzed healthy volunteers can be found in Table 1.

Table 1: Volunteer characteristics

	Session 1 (n=9) Median [IQR]	Session 2 (n=9) Median [IQR]
Plaster application time (min)	365 [360-450]	434 [425-455]
Blood pressure systole (mmHg)	123 [120-125]	129 [124-130]
Blood pressure diastole (mmHg)	84 [78-85]	85 [80-90]
Body length (cm)	184 [173-190]	178 [173-181]
Weight (kg)	84 [65-90]	77 [71-80]
Age (years)	28 [26-30]	28 [23-32]
Gender (female)	22%	33%

3.1 Zero and arterial blood gas oxygen tension validation

While the arm was in the incubator with an oxygen tension < 5 mmHg, the blood supply to the arm was occluded and pressure was applied to the skin sensor to approximate zero oxygen conditions. In all cases mitoPO$_2$ dropped and reached an equilibrium. The lowest measured mitoPO$_2$, median [IQR] minimum value was 3.0 [1.0 – 4.0] mmHg. In one case the steady state did not go below 5 mmHg and reached an equilibrium at 15 mmHg, seen in Figure 4a.

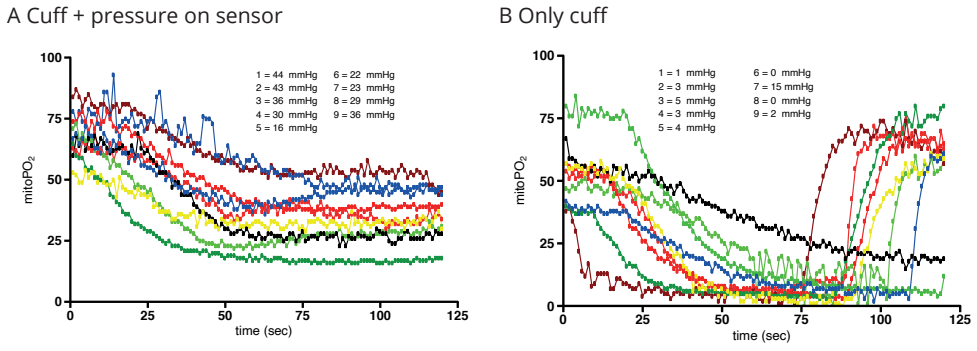


Figure 4: *A) mitoPO$_2$ measurements in the incubator with pressure applied to the COMET skin sensor and an inflated upper arm cuff, pressurized to 50 mmHg above systolic blood pressure to stop the arterial flow in the arm. The listed values are the individual minimum mitoPO$_2$ levels just before the pressure is released.*
B) mitoPO$_2$ measurements of the arm in which only the cuff around the upper arm was pressurized to 50 mmHg above systolic blood pressure to stop the arterial blood flow in the arm. The listed mitoPO$_2$ values are the equilibrium value at the end of the measurement.

The additional effect of local pressure on the tissue with the COMET skin sensor on the deoxygenation kinetics compared to use of only a blood pressure cuff can clearly be seen in Figure 4. In the experiments shown in Figure 4b only an upper arm cuff was pressurized to stop the arterial blood flow. As a result, the mitoPO$_2$ stabilized at median of 30.0 [24.5 – 36.0] mmHg, greatly contrasting with the low mitoPO$_2$ 3.0 [1.3 – 4.8] mmHg when pressure is also applied on the skin sensor itself. If pressure is applied to the skin sensor the decrease in oxygen tension is faster, 2.1 [1.0 – 2.9] mmHg/s compared to 1.3 [1.2 – 1.4] mmHg/s without pressure.

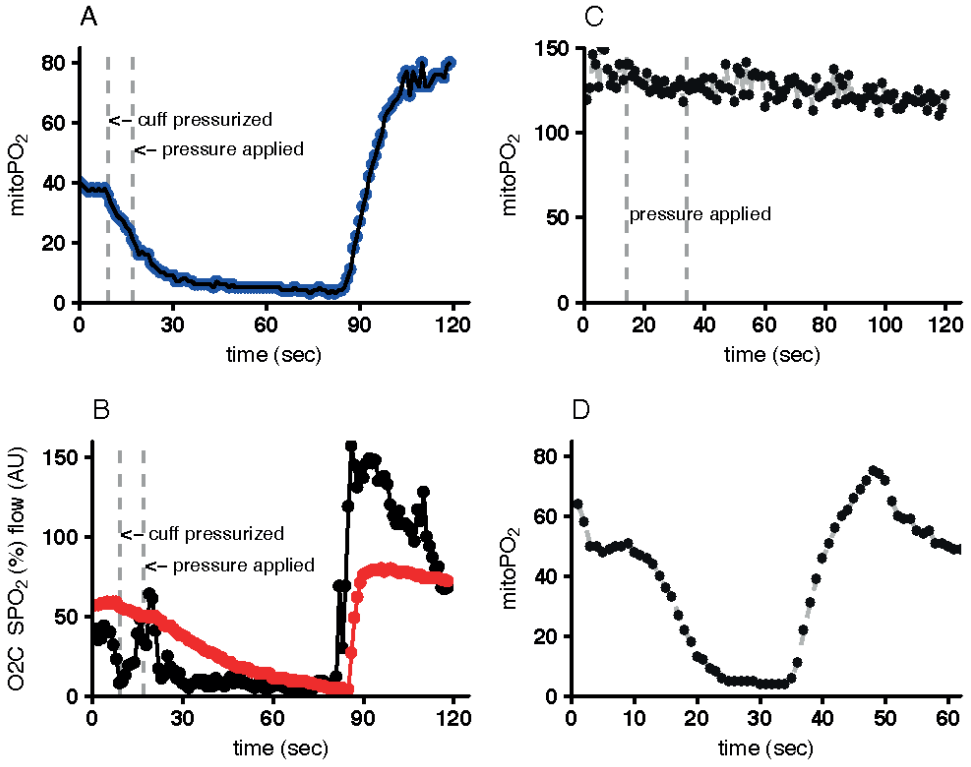


***Figure 5**: **A)** MitoPO$_2$ baseline measurement, at t=9 (sec) blood pressure cuff inflated to 50 mmHg above systolic blood pressure, t=17 (sec) manual pressure on the COMET skin sensor, at t=84 (sec) release of pressure from skin sensor and cuff. **B)** O2C microvascular blood flow (black) and tissue oxygenation SpO$_2$ (red) with the pressure cuff applied and pressure on the skin sensor. **C)** Cyanide was applied to the skin to block mitochondrial respiration. While pressure was applied no sudden drop was seen, indicating cyanide-induced blockage of mitochondrial respiration. **D)** Ten minutes after cyanide application a mitochondrial respirational check was done.*

Application of cyanide cream on the skin in the first session led to low signal quality and, except for one case, mitoPO$_2$ readings well below the corresponding PaO$_2$ values (Figure 6a). This COMET behavior was analyzed in cooperation with the manufacturer and appeared to be caused by the detector gating. Due to the specific timing of this gating, the very short delayed fluorescence lifetimes resulting from the high intracellular PO$_2$ were omitted from the signal analysis, leading to an erroneously low steady mitoPO$_2$ around 66 mmHg.

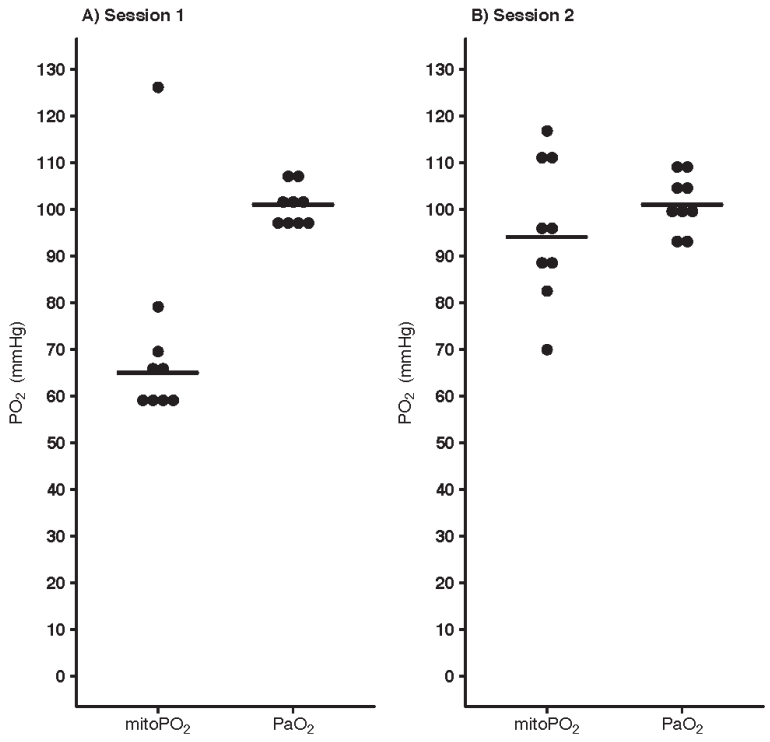


Figure 6: *Comparison between COMET mitoPO$_2$ after cyanide cream had been applied to block mitochondrial respiration and arterial blood gas PaO$_2$ taken from a radialis.* ***A)*** *Data from session 1, even though the delayed fluorescence was insufficient a mitoPO$_2$ was displayed around 65 mmHg. One subject with a strong signal the short lifetime was measurable, seen as black dot 126 mmHg.* ***B)*** *Data from session 2 with adjusted software. No significant difference was seen in session 2 between mitoPO$_2$ and PaO$_2$.*

To enable accurate detection of high mitoPO$_2$ values the timing of the gating was adjusted by a temporary adaptation in the firmware. During session 2 data was collected with this adjusted software. Now, the median [IQR] mitoPO$_2$ was 94.1 mmHg [87.2 – 110.9] and

did not differ significantly (p=0.5) from the sampled PaO_2 of 101.0 mmHg [98.0 – 106.0]. When pressure was applied to the skin sensor the $mitoPO_2$ did not decrease, indicating the absence of mitochondrial respiration, as shown in Figure 5c. Within all subjects the mitochondrial respiration returned after approximately 15 minutes. No major adverse events caused by the cyanide application were witnessed. Apart from a temporarily red skin, no pain, skin irritation or other effects of the cyanide cream were reported.

3.2 Comparison of monitors

Arterial occlusion of the arm led to an immediate decline and subsequent stop of microcirculatory blood flow measured by the O2C. $MitoPO_2$ and tissue oxygen saturation followed. A linear regression of the measured decline during the arterial occlusion with the cuff provided different slopes for all measurements as shown in Table 2. An example of measurements is shown in Figure 7A, with the mean of the z score of all subjects shown in Figure 7B.

Table 2: Linear fit of arterial occlusion of the arm with a pressurized cuff

	Baseline value Median [IQR]	**Decline Mean ± SD**	**z-score* Decline Mean**
$MitoPO_2$ (mmHg)	68 [61-76.5]	-0,75 ± 0,06	-0,089
Flow O2C (FU)	35 [20-57]	-2,30 ± 0,37	-0,15
sat O2C (%)	49 [43-65]	-0,51 ± 0,05	-0,062
NIRS (%)	76 [72-82]	-0,21 ± 0,04	-0,058
$TcPCO_2$ OxiVenT (mmHg)	51 [47-56]	$-0{,}37*10^{-3}$ ± 0,03	-0,010
Heating OxiVenT (mWatt)	130 [123-142]	$-0{,}95*10^{-1}$ ± 0,06	-0,050

*z-score = (data point-average)/standard deviation

Interestingly the $tcPCO_2$ measurement was stable during this short arterial stop, although the heating power required to maintain a stable sensor temperature of 43 °C changed slightly. After a relatively long delay an increase in $tcPCO_2$ was seen.

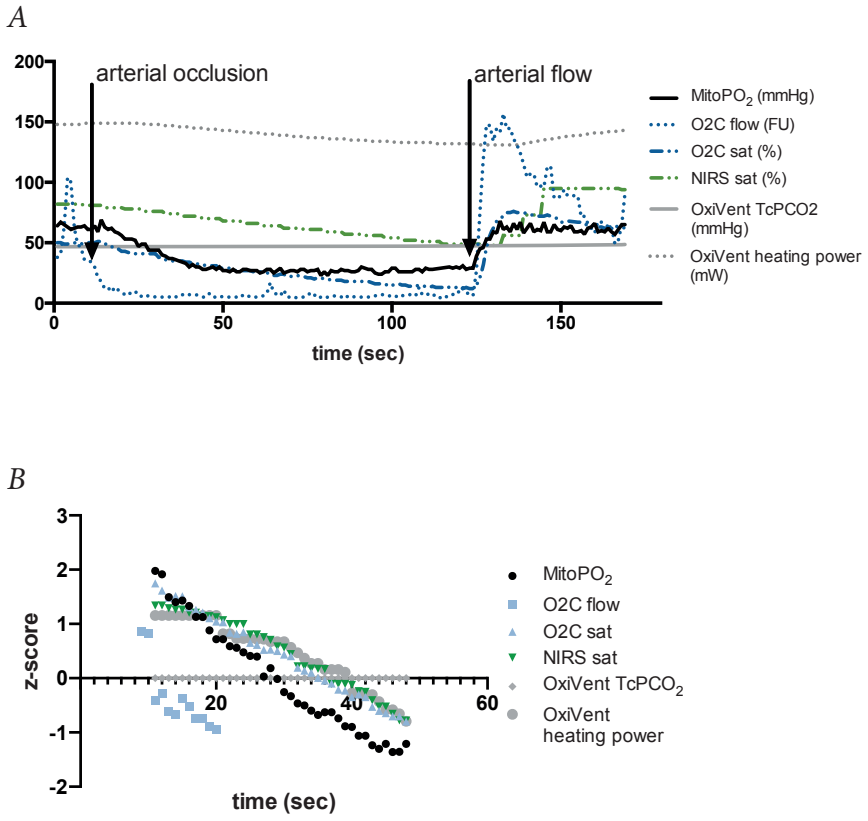


Figure 7: *A)Typical example of arterial occlusion of the forearm. At t=10 seconds the blood pressure cuff was inflated to 50 mmHg above the blood pressure measured earlier. At t=120 seconds the blood pressure cuff was released and arterial flow returned. B) Average data of 9 subjects. Data shown in z-score transformation, z=(data point – mean)/standard deviation to compare the overall decline part of the data.*

4. Discussion

This study demonstrates that the calibration of COMET device, originally determined in animal experiments, is adequate for cutaneous mitoPO$_2$ measurements in man. Furthermore, dynamic oxygenation measurements aimed at gaining insight in tissue oxygen consumption depend on the specific measuring technique and method to cease oxygen supply to the measurement spot.

The COMET measurement system measures relatively high mitoPO$_2$ values compared to the general idea that mitoPO$_2$ should be very low in order to drive oxygen diffusion [6, 18, 19]. This study shows that such high mitoPO$_2$ values are not the result of an

inadequate calibration. On the contrary, in this study the COMET showed the tendency to underestimate mitoPO$_2$ in the case intracellular PO$_2$ was artificially increased to arterial oxygen levels.

The mitoPO$_2$ measured with the COMET monitor is higher than expected. MitoPO$_2$ appears to be, depending on the measurement site and respiratory rate of the tissue, much closer to microvascular oxygen tension [20, 21], and thus closer to tissue and/or interstitial oxygen levels [22, 23], than anticipated [7]. There are several reasons why mean mitoPO$_2$ in a tissue sample cannot be an order of magnitude lower than microvascular and interstitial oxygen tension; First, oxygen does not disappear stepwise but gradual, so several mitochondria will see aPO$_2$ close to intravascular values. Second, oxygen diffuses also from large vessels so contribute to cellular oxygen delivery [24], so several mitochondria have a higher PO$_2$ than capillary oxygen tension. Third, the mitoPO$_2$ will not be substantially lower than interstitial PO$_2$ because the oxygen gradient over the cell membrane is small [1]. Typically reported baseline mitoPO$_2$ values are 40-70 mmHg. Other oxygen measurements in the skin are matching these values [23]. Importantly, it has been demonstrated in both a preclinical [25] and clinical setting [4] that mitoPO$_2$ provides different information than hemoglobin saturation-based techniques like near-infrared spectroscopy.

Application of cyanide on the skin led to a temporary block of mitochondrial respiration and abolishment of the oxygen gradient. In the first series of 9 investigated subjects, the timing between the laser pulse and the end of the off-gating of the photomultiplier (PMT) in the COMET proofed too long to adequately detect the short delayed fluorescence lifetimes caused by the artificially high intracellular PO$_2$. The gating itself is necessary to prevent damage to the sensitive detector due to laser light and prompt fluorescence [4], and its timing is a trade-off between several factors, foremost the ability to accurately measure high mitoPO$_2$ (supraphysiological) and protecting the detector. After adjustment of this timing in the firmware, PMT gating interference was sufficiently reduced to allow collection of the delayed fluorescence signal after topical application of cyanide. Due to this adaptation, we were able to demonstrate that mitoPO$_2$, as measured with COMET, corresponds well to PaO$_2$ in the absence of mitochondrial oxygen consumption. Under more physiological circumstances the timing of the PMT gating is much less critical as delayed fluorescence lifetimes are longer and easier to detect. COMET measured very low mitoPO$_2$ after oxygen deprivation and overall the calibration of the device seems adequate for its purpose.

This study also presents the comparison of different oxygen consumption measurements in the arm. The COMET was compared to O2C, INVOS and SenTec OxiVenT™ during an arterial occlusion test in nine subjects. During arterial occlusion all oxygenation parameters show a decline in a comparable rate. Also, the measured decline in NIRS saturation of 0.21%/sec (12.6%/min) in this study is comparable to previously found values of 10.8, 13.2, 22.8%/min during occlusion of an extremity [26]. However, during a dynamic measurement for measuring mitochondrial oxygen consumption (mitoVO$_2$), with pressure on the measurement probe, a faster decline is seen. This decline is seen in all but one subject in figure 4 A with an equilibrium at 15 mmHg. Since the curve of this individual shows similarities to the measurements without local pressure on the probe, we think that probably the effect of local pressure on the sensor was inadequate. We hypothesize that in this case an equilibrium emerges between the still saturated hemoglobin and mitochondrial respiration, similar to the situation in laboratory animals [27]. Also, when a mitoVO$_2$ procedure is done on the sternum we do not see such equilibrium at a high mitoPO$_2$ value. Therefore, we think that the pressure on the skin sensor did not adequately push away the erythrocytes in the measurement volume.

MitoVO$_2$ measurements should preferably be done on skin above a bone structure to allow the buffer of erythrocytes to be pushed away. The arm is therefore not the preferred site because the skin is not located above a flat bone. This likely resulted in a relatively slow median mitoVO$_2$ of 2.1 mmHg/s in comparison to measurements done on the sternum with a median mitoVO$_2$ 5.8 mmHg/s measured on healthy volunteers in our lab [10]. When the skin sensor is on top of a bone structure, with a little pressure the microcirculation is blocked and the erythrocytes are pushed out of the measurement volume. In this study the applied pressure was not measured but this could add to the standardization of a mitoVO$_2$ maneuver and improve the repeatability. However, arterial occlusion tests can only be done on an extremity and therefore the mitoVO$_2$ values are different from other healthy volunteer studies [6, 10]. Whilst the forearm is not a preferred measurement site a large difference in mitoVO$_2$ could be demonstrated if pressure is exerted onto the COMET Skin Sensor, 2.1 mmHg/s compared to 1.3 mmHg/s during arterial occlusion. The oxygen buffer available in microcirculation likely accounts for the difference of 0.8 mmHg/s.

Both the mode of measurement (hemoglobin-based versus non-hemoglobin-based) and differences in tissue penetration depth per technique might account to the observed differences in oxygen disappearance rates. The COMET has a penetration depth of less than a mm, in contrast to infra-red optical techniques (>900nm wavelength) with

a penetration depth of several cm. Since O2C and INVOS measure deeper in the tissue, and thus in a different tissue compartment, the decline in oxygen saturation represents a larger measurement volume. After the occlusion an equilibrium emerges between the available oxygen, mainly dependent on the concentration and amount of arterial and venous hemoglobin available in the vessels, and mitochondrial respiration. It is not possible to push away or largely reduce the number of erythrocytes in the infra-red measurement volume. A large measurement volume that contains erythrocytes without the ability to reduce the oxygen buffer results in a slower decrease in saturation. When performing a dynamic measurement with the COMET local pressure with the Skin Sensor leads to largely eliminating the available erythrocytes from the microcirculation and therefor the dynamic measurement may be less dependent on the availability of the oxygen buffer.

5. Conclusion

This study shows that mitochondrial oxygen partial pressures measured with Pp-IX lifetime technique are comparable to the arterial PaO_2 during blockade of mitochondrial respiration with topical application of cyanide. Therefore, this study demonstrates that the calibration of COMET device, originally determined in animal experiments, is valid in human cutaneous mitoPO_2 measurements.

For mitochondrial oxygen consumption measurements not only blood flow occlusion, but applying pressure on the COMET Skin Sensor is of great importance to clear the measurement site of available oxygen-carrying erythrocytes. Without a technique to eliminate this oxygen buffer the consumption measurement underestimates the actual mitochondrial oxygen consumption.

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Chapter 5

Non-invasive versus ex vivo measurement of mitochondrial function in an endotoxemia model in rat: toward monitoring of mitochondrial therapy

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Abstract

Mitochondrial function has been predominantly measured *ex vivo*. Due to isolation and preservation procedures *ex vivo* measurements might misrepresent *in vivo* mitochondrial conditions. Direct measurement of *in vivo* mitochondrial oxygen tension (mitoPO$_2$) and oxygen disappearance rate (ODR) with the protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT) might increase our understanding of mitochondrial dysfunction in the pathophysiology of acute disease.

LPS administration decreased mitochondrial respiration (ODR) *in vivo* but did not alter mitochondrial function as assessed with *ex vivo* techniques (high resolution respirometry and specific complex determinations). PpIX-TSLT measures *in vivo* mitoPO$_2$ and ODR and can be applied non-invasively at the skin.

Key words: Mitochondria, Endotoxemia, Lipopolysaccharides, Succinic Acid, 5-aminolevulinic acid.

Introduction

Acute changes in mitochondrial function may play a role in the pathophysiology of sepsis (Fink, 2002). However, the options for monitoring mitochondrial function in patients are limited since only *ex vivo* techniques are available. The most common *ex vivo* technique measures oxygen consumption using oxygen electrodes (Estabrook, 1967), such as high-resolution respirometry (Gnaiger et al., 1995). Respirometry measures oxygen consumption in suspensions of isolated cells, isolated mitochondria or homogenates of small tissue biopsies and, therefore, might not adequately reflect the *in vivo* situation in acute changes of mitochondrial function (Jeger et al., 2013). A new non-invasive method to measure mitochondrial function could therefore improve the diagnosis of sepsis, and maybe even open the door to new therapies for which there is an urgent need (Jeger et al., 2013).

Techniques to study mitochondrial function *in vivo* are the Nuclear Magnetic Resonance (NMR)-technique and NADH fluorometry (Fosslien, 2001). NMR and NADH fluoroscopy both show determinants of metabolic state. The current inability of bedside monitoring and its costs make NMR less suitable for clinical use.

Changes of mitochondrial metabolic states measured by NADH fluoroscopy have been shown in a research setting. Despite these results, standard clinical monitoring of mitochondrial function by NADH fluoroscopy is not yet an option due to its sensitivity to artifacts and difficult interpretation.

An innovative method to monitor mitochondrial function *in vivo* has been developed and evaluated in our laboratory (Harms et al., 2013). The protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT) enables measurement of mitochondrial oxygen tension (mitoPO_2) in living cells and tissues (Mik et al., 2008; 2006). Further development of this technique allowed the detection of mitoPO_2 and mitochondrial oxygen disappearance rate (ODR) in the skin *in vivo* (Harms et al., 2013; 2012; 2011). Recently, the COMET monitor (Photonics Healthcare B.V., Utrecht, The Netherlands) has become commercially available. This device allows measurements of mitoPO_2 and ODR non-invasively in healthy volunteers and patients at the bedside based on PpIX-TSLT (Baumbach et al., 2018; Ubbink et al., 2016).

In two previous studies with the PpIX-TSLT we have measured a decreased ODR in the skin of LPS-induced rats (Harms et al., 2015a; 2015b). The effect of LPS administration

causes macrohemodynamic and microcirculatory changes and inhibits mitochondrial complex I (Choumar et al., 2011). Until now, we did not directly compare an *ex vivo* muscle biopsy with our *in vivo* technique. This study therefore aims to perform this direct comparison of *in vivo* and *ex vivo* data in a clinically relevant model of sepsis.

Monitoring changes in ODR with the PpIX-TSLT during endotoxemia, as a model for sepsis, is promising. Showing changes in *in vivo* mitochondrial function due to an intervention would strengthen the PpIX-TSLT as a monitor. In classic respirometry the substrate succinate is used to study mitochondrial oxygen consumption linked to electron flow through complex II (Silva and Oliveira, 2011). In isolated mitochondria from endotoxemic rats the addition of succinate resulted in a normalization of mitochondrial oxygen consumption (Protti et al., 2007). We therefore measured with PpIX-TSLT whether succinate had similar effects on the mitochondrial respiratory chain *in vivo* in a rat model of endotoxemia with succinate pre-treatment.

Until now, two important questions remained unanswered. The first question is how our PpIX-TSLT measurements in skin relate to *in vi*vo and *ex vivo* measurements in muscle, as the standard tissue for mitochondrial biopsies and measurements (experiment A). The second question is whether measurements performed with PpIX-TSLT besides detection of gross changes in mitochondrial respiration, are also sensitive enough to monitor therapeutic effects (experiment B). Successful *in vivo* measurement of mitochondrial parameters could help unravel the pathophysiology involved in critical illness. Additionally, it would provide a new technique for guiding therapies aimed at improving mitochondrial function.

Material and Methods

Subjects and preparation

The experimental protocols (A; DEC 129-14-03 and B; DEC 129-12-11) were approved by the Animal Research Committee of the Erasmus University Medical Center Rotterdam. Animal care and handling were performed in accordance with the guidelines for Institutional and Animal Care and Use Committees.

For this study, 54 male Wister rats (Charles River, the Netherlands; body weight 280-350 g) were used, 14 rats in protocol A (age 77-111 days) and 40 rats in protocol B (age 59-73 days). Anesthesia was induced by an intraperitoneal injection of a mixture of ketamine 90 mg kg^{-1} (Alfasan, Woerden, the Netherlands),

medetomidine 0.5 mg kg^{-1} (Sedator, Eurovet Animal Health BV, Bladel, the Netherlands) and atropine 0.05 mg kg^{-1} (Centrofarm Services BV, Etten-Leur, the Netherlands). Tracheotomy was performed to enable mechanical ventilation. Ventilator settings were adjusted on end-tidal PCO_2, keeping the arterial CO_2 partial pressure between 35-45 mmHg; the inspired oxygen concentration was set at 40%. The right jugular vein was catheterized with a polyethylene 0.9 mm catheter for intravenous fluid administration. The left femoral artery was catheterized to monitor arterial blood pressure and heart rate, and for hourly blood gas analysis. Cardiac output was measured by a thermodilution method with a thermistor inserted in the right carotid artery. Anesthesia and fluid balance were maintained by continuous infusion of ketamine (50 mg kg^{-1} h^{-1}), a crystalloid (Ringer's, B. Braun Melsungen AG, Melsungen, Germany) and a synthetic colloid solution (2.5 mL kg^{-1} h^{-1}) (Voluven®, Fresenius Kabi, Bad Homburg, Germany). Body temperature was measured rectally and maintained at 38 ± 0.5°C by means of a heating pad.

Experimental procedures

Abdominal hair was removed by shaving followed by application of commercially available hair removal cream (Veet, Reckitt Benckiser Co., Slough, UK) for about 5 min. PpIX was induced by applying a freshly prepared 2.5% 5-aminolevulinic acid (ALA) (Sigma-Aldrich, St. Louis, MO, USA) in hydrophilic cremor lanette (Lanettecreme I FNA, Bipharma, Weesp, the Netherlands). The skin was covered with aluminum foil to protect PpIX from light exposure.

Experiment A; With muscle measurement

The skin above the m. quadriceps femoris was removed. The ALA applied on the skin and muscle were covered with an adhesive film to avoid oxygen diffusion from the surroundings. The ALA applied tissue was covered with aluminum foil to protect PpIX from light exposure.

The 14 rats were divided into 2 groups; a time-control group (M-TC, N=6) and a LPS-induced endotoxemic group (M-LPS, N=8). TC were matched on duration of the experiment, a lactate concentration above 2 mmol/l in the blood gas analysis was used as a marker for the second ODR measurement in the LPS group. Fluid resuscitation (Voluven®, 5 ml*kg-1*h-1) was given to prevent hemodynamic shock and a decline in

mitoPO$_2$. In the LPS group endotoxemia was induced by intravenous LPS injection (4.5 mg/kg lipopolysaccharide from E.Coli 0127:B8, Sigma-Aldrich, St. Louis, MO, USA). After recording baseline values (T0), a solution of 1 mg/ml LPS was infused during 30 min. Fluid resuscitation was performed by doubling the maintenance colloid infusion directly after LPS application. The timeline of this experiment is shown in figure 1A.

Experiment B; Without muscle measurement

The 40 rats were randomly divided into 5 groups (8 rats/group); two control groups consisting of a time control group (TC) and a control group receiving methyl-succinate (SC). Three LPS-induced endotoxemic groups consisting of a group in which only LPS was given (LPS --), a LPS group receiving fluid resuscitation (LPS+-), and a LPS group receiving fluid resuscitation and methyl-succinate (LPS++). Fluid resuscitation (Voluven®, 5 ml*kg^{-1}*h^{-1}) was given to prevent hemodynamic shock and a decline in mitoPO$_2$. Succinate dimethyl ester (Brunschwig Chemie, Amsterdam, the Netherlands) was infused (concentration 0.67 M, rate 5 ml*kg^{-1}*h^{-1}) 2h prior to the LPS infusion in the SC and LPS ++ groups. For the remaining groups, the succinate solution was exchanged for saline at the same rate.

In all three LPS groups endotoxemia was induced by intravenous LPS injection (3mg/kg lipopolysaccharide from E.Coli 0127:B8, Sigma-Aldrich, St. Louis, MO, USA). After recording baseline values (T0), a solution of 1 mg/ml LPS was infused during 15 min. Fluid resuscitation was performed by doubling the maintenance colloid infusion directly after LPS application and by an additional fluid bolus of 1 ml during 10 min prior to T1. The timeline of the experiment is shown in Figure 1B.

Principle of MitoPO$_2$ and oxygen disappearance measurements

The background of the PpIX-TSLT is described in detail elsewhere (Mik et al., 2008; 2006). In short, PpIX is the final precursor of heme in the heme biosynthetic pathway. PpIX is synthesized in the mitochondria, ALA is the rate-limiting step in this pathway and therefore administration enhances the PpIX concentration substantially. PpIX possesses a triplet state that reacts strongly with oxygen, making its delayed fluorescence lifetime oxygen-dependent according to the Stern-Volmer equation (Mik et al., 2002).

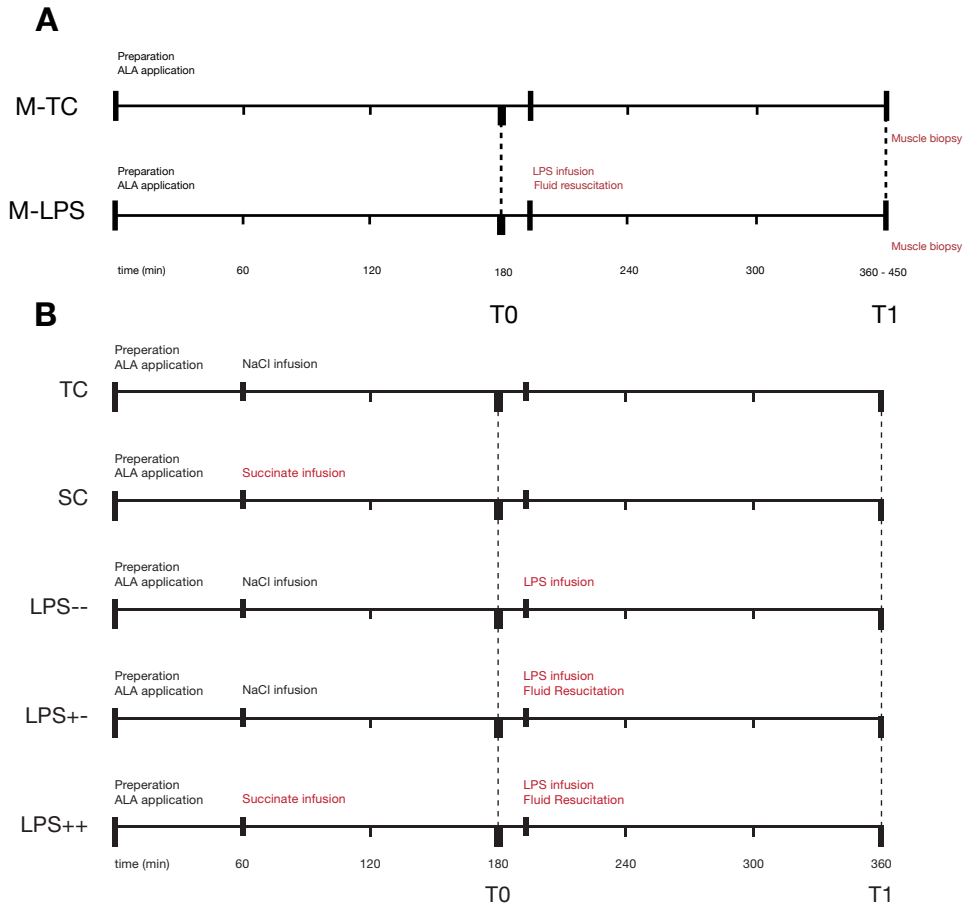

Figure 1. Schematic timeline of the experimental protocol. Panel A: Experiment A. with muscle measurement. M-TC: time control, M-LPS: endotoxemia with fluid resuscitation. Panel B: Experiment B. without muscle measurement. TC; time control, SC; succinate control, LPS --; endotoxemia, LPS +-; endotoxemia with fluid resuscitation, LPS ++; endotoxemia with fluid resuscitation and succinate. ALA; 5- aminolevulinic acid, LPS; lipopolysaccharide, T0 and T1 are the time points of mitoPO2 and ODR measurement.

The oxygen disappearance rate is measured directly after local occlusion of the oxygen supply. The reflection probe was mounted on a height-adjustable frame, above the ALA-treated skin, providing different settings of the probe distances to the skin. Local occlusion of the microcirculation in the skin was obtained by local pressure with the measurement probe. This simple procedure created reproducible stop-flow conditions and induced measurable oxygen disappearance rates, due to cessation of microvascular oxygen supply and ongoing cellular oxygen consumption. MitoPO_2 was measured before,

during and after application of pressure at an interval of 1Hz and using 1 laser pulse per measurement. We have described the fundamental principles behind the technology and have provided a working implementation of the technique for ODR measurements *in vivo* (Harms et al., 2013) and a method to calculate ODR from the mitoPO$_2$ kinetics. On each time point mitoPO$_2$ and ODR are presented as average of 4 replicate measurements. In the time control group at T1 a starting mitoPO$_2$ of around 50 mmHg was chosen, to match the LPS group, for fitting of the ODR. This was done to prevent potential negative effects of increased noise at higher mitoPO$_2$ readings on ODR.

Mitochondrial assay in muscle biopsies

At the end of the experiment a muscle biopsy was taken from the m. quadriceps femoris. The biopsy was transferred to mitochondrial respiration buffer (MRB, a hybrid buffer consisting of 110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 10 mM monobasic potassium phosphate, 3 mM magnesium chloride, 20 mM HEPES, 1 mM EGTA, and 0.1% (w/v) BSA at pH 7.1 at 37°C) on ice, homogenized using a Potter-Elvehjem PTFE pestle and glass tube, and injected in the respirometer. A small piece of muscle was snap-frozen in liquid nitrogen and stored at -80 C for later determination of complex concentration and activity.

High-resolution respirometer

Oxygen consumption was measured using a high-resolution respirometer (Oxygraph O2k, Oroboros, Innsbruck, Austria). Prior to homogenate loading the instrument was calibrated following the manufacturer instructions and loaded with 2.1 ml MRB. An oxygen solubility in water of 0.92 was used to calculate oxygen levels. 0.1 ml of muscle homogenate was added to the respirometer, and the chamber was closed. All chemicals for the respirometer experiments were obtained from Sigma-Aldrich (Darmstadt, Germany). To determine mitochondrial complex activity, first pyruvate (5mmol/l final concentration) and malate (2mmol/l final concentration) were added. Subsequent addition of ADP (0.25 mmol/l final concentration) showed full activity of complex 1. After addition of rotenone (0.5 micromol/l final concentration) to the chamber complex 1 activity was stopped and succinate (5 mmol/l final concentration) was added to measure complex 2 activity. The ATPase was inhibited by adding oligomycin (2.5 micromol/l final concentration) and subsequently FCCP (1 mmol/l solution) titration was performed to determine maximal oxygen consumption. The oxygen consumption measured by the high-resolution respirometer was corrected for citrate synthase activity, an indicator

of the number of mitochondria was measured according to Srere (1969) (Srere and Lowensteijn, 1969) of the homogenate to enable objective comparison between samples.

Complex determination

To obtain an indication of the function of the oxidative phosphorylation the activity of citrate synthase (CS) and the activity of complex 1 and 2 were analyzed. Activities of the complexes were based on the amount of CS to make objective comparison possible.

Tissue homogenates were prepared from frozen muscle in 0.25 M sucrose, 10 mM N-[2-hydroxyethyl] piperazine-N'-[2 ethylsulfonic acid](HEPES) and 1 mM Ethylene Diamine Tetraacetic Acid (EDTA), pH 7.4. Enzyme activities were measured in the homogenates. Activities of the complexes of the mitochondrial respiratory chain were determined in muscle homogenates by spectrophotometric methods as described before (Scholte et al., 1995) Complex I or NADH-Coenzyme Q reductase was determined kinetically by following the rotenon sensitive decrease in the amount of NADH. Complex II or Succinate Coenzyme Q reductase was measured by following the TTFA dependent reduction of DCPIP after addition of succinate. Citrate synthase was used as an indicator for the number of mitochondria to enable an objective comparison.

Statistical analysis

In experiment A data are expressed as median with interquartile range. For intragroup analysis the Wilcoxon matched pair signed rank test was used, for intergroup analysis the Mann-Whitney test was used. For experiment B data are expressed as means with standard deviation (SD), unless stated otherwise. A paired t-test was used to detect intragroup differences. Two-way ANOVA with repeated measurements, using post hoc multiple comparison with Bonferoni correction, was used to detect intergroup differences. For the hemodynamic data only intergroup differences were tested compared to time control. Normality was tested by Q-Q analysis and the Shapiro-Wilk test. A p-value < 0.05 was considered statistically significant. Statistical calculations were performed using Graphpad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

Results

Experiment A

Hemodynamic parameters

Table 1 presents data on hemodynamic parameters of experiment A. At baseline (T0) all rats were hemodynamically stable and no significant difference was observed between the groups. Three hours after LPS infusion heart rate (p=0.0293) and lactate (p=0.0007) increased compared to the time-control group.

Table 1
Hemodynamic parameters experiment A.

	T0	T1
MAP (MmHg)		
M−TC	95 [14]	80 [6]
M−LPS	93 [6]	89 [26]
Cardiac output (ml/min)		
M−TC	118 [39]	142 [67]
M−LPS	148 [60]	167 [60]
Heart rate (bpm)		
M−TC	286 [41]	271 [42]
M−LPS	284 [37]	314 [56][a]
Lactate (mmol/L)		
M−TC	1.1 [0.1]	0.9 [0.4]
M−LPS	0.9 [0.1]	2.4 [0.6][b]

Values are median [IQR], M−TC, time control; M−LPS, endotoxemia + fluid resuscitation.
[a] = $P < 0.05$ compared to TC.
[b] = $P < 0.001$ compared to TC.

MitoPO$_2$ measurements

In accordance with previous experiments (Harms et al., 2015a), the application of ALA increased the amount of PpIX in the mitochondria to detectable levels in all animals. MitoPO$_2$ could be measured in the abdominal rat skin for the duration of the experiment (3 hours after application of ALA). MitoPO$_2$ in the abdominal skin didn't change for 3 hours (T0 → T1) in both the time control (M-TC) and endotoxemia (M-LPS) group (Fig 2A). However, muscle mitoPO$_2$ at T1 in the M-TC group was markedly higher, 82 [42] mmHg vs 46 [27] mmHg, compared to the M-LPS group (Fig 2B).

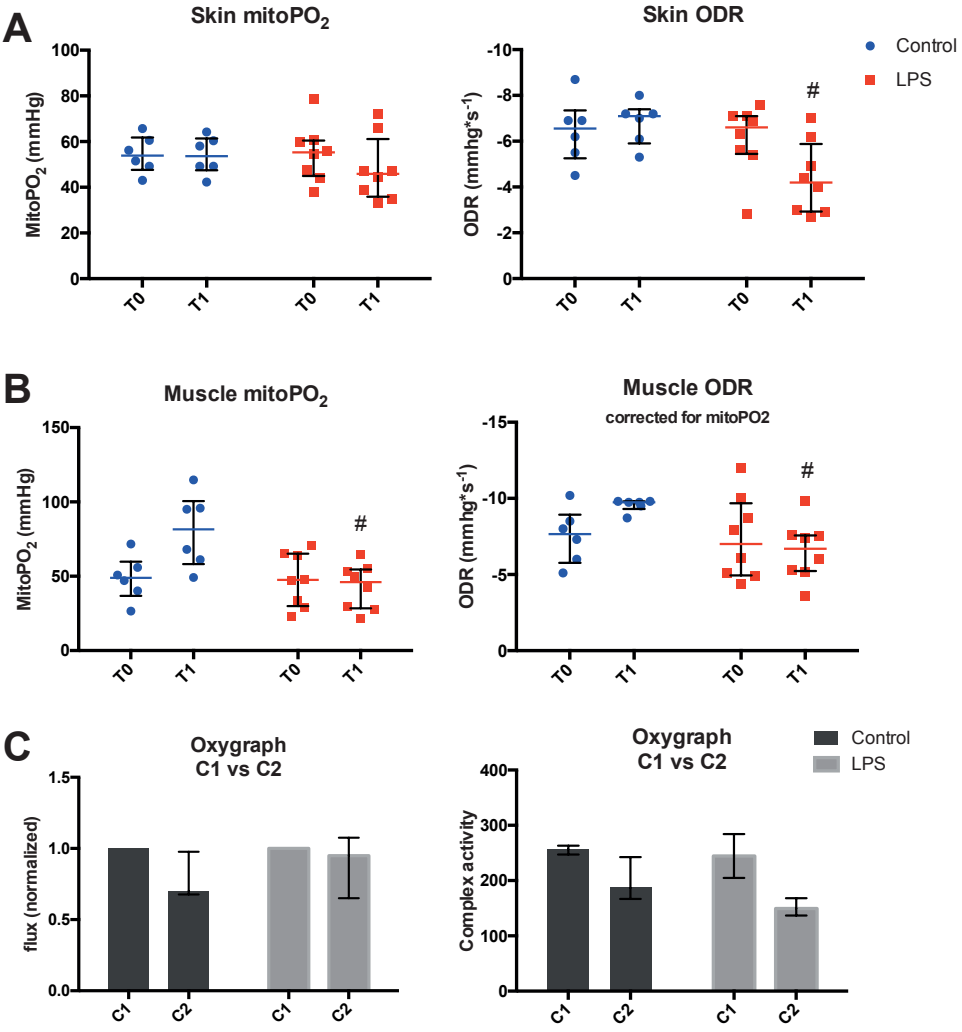

Figure 2. Panel A: $MitoPO_2$ and ODR measurements in skin at T0 and T1 in the different experimental groups, ODR M-LPS (T1) vs M-TC (T1) (p=0.0093) Panel B: $MitoPO_2$ and ODR in muscle at T0 and T1 in the different experimental groups, $mitoPO_2$ M-LPS T1 versus M-TC T1 (p=0.127) and ODR decreased M-LPS T1 vs M-TC T1 (p=0.0127). Panel C: comparison of flux (normalized) between complex 1 and complex 2 corrected for protein concentration, and comparison of complex activity in the snap frozen muscle biopsy corrected for citrate synthase. TC; time control, LPS; endotoxemia with fluid resuscitation, LPS; lipopolysaccharide, ODR; mitochondrial oxygen disappearance rate. T0 and T1 are the time points of ODR measurement. Data are presented as median ± IQR, * significant difference compared to baseline measurement, # significant difference compared to time control measurement ($p<0.05$); (TC; 6 rats, LPS; 8 rats).

ODR measurements

ODR was derived from the mitoPO$_2$ slope during blockage of microcirculatory blood flow. At T0 the initial mitoPO$_2$ was approximately 60 mmHg. Blocking the microcirculation by local pressure with the measurement probe caused a drop in mitoPO$_2$ from 55 mmHg to approximately 10 mmHg in 10 s, this resulted in a ODR of 5.8 mmHg*s^{-1} (Fig. 3). In experiment A, at baseline no difference was found in skin and muscle between M-TC and M-LPS group (figure 2A and B). In contrast, at T1, a significant lower value was measured in skin (LPS; -4.2 [3.0] mmHg*s^{-1} vs TC; -7.1 [1.5] mmHg*s^{-1}) and in muscle (LPS -6.7 [2.4] mmHg*s^{-1} vs TC -9.7 [0.5] mmHg*s^{-1}) (Fig. 2B).

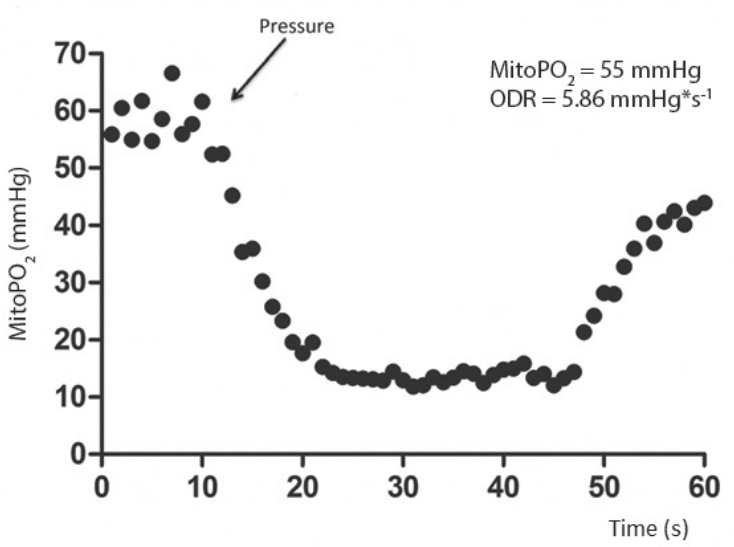

Figure 3. Typical example of *in vivo* mitochondrial respirometry measured at T0 on the abdominal skin of a rat. The ODR was determined from the linear part of the oxygen disappearance curve by fitting equation 2. MitoPO$_2$ was the mean mitoPO$_2$ before the start of tissue compression.

Mitochondrial assay in muscle biopsies

Muscle biopsies were performed at the end of the experiment. We found no difference in complex 1 and 2 activity in the high-resolution respirometer of the homogenized muscle in both groups, as shown in figure 2C. We found no difference in complex activity corrected for citrate synthase activity measured in the snap frozen biopsies.

Experiment B

Hemodynamic parameters

At baseline (T0) all rats were hemodynamically stable and no significant difference was observed between the groups, as shown in table 2. Three hours after LPS infusion (T1), significant hemodynamic changes were observed in the LPS groups, reflected by an elevation in serum lactate levels in all three endotoxemia groups. A decrease in mean arterial blood pressure (MAP) was seen in the LPS -- and the LPS ++ group compared to TC, but not in the LPS + - group. Cardiac output decreased in the LPS -- (p=0.049) and LPS ++ (p=0.039) groups (102 ± 59 and 100 ± 39 bpm vs 140 ± 56 bpm TC) compared to time control. The succinate control group showed a significant decrease in heart rate (p=0.0245), 267±11 vs 290±13 bpm, compared to time control.

MitoPO_2 measurements

A mitoPO_2 value around 60 mmHg was observed for all experimental groups at T0. In the LPS -- group a significant decline in mitoPO_2 from 62 ± 9 mmHg (T0) to 41 ± 12 mmHg (T1) was measured. At T1 a significant lower value of mitoPO_2 was measured in the LPS -- and LPS +- (p=0.0006) groups compared to time control, of 41 ± 12 mmHg (LPS --) and 54 ± 21 mmHg (LPS +-) compared to 74 ± 17 mmHg (TC) respectively (Fig. 4A). The individual changes in mitoPO_2 are presented in Figure 4B.

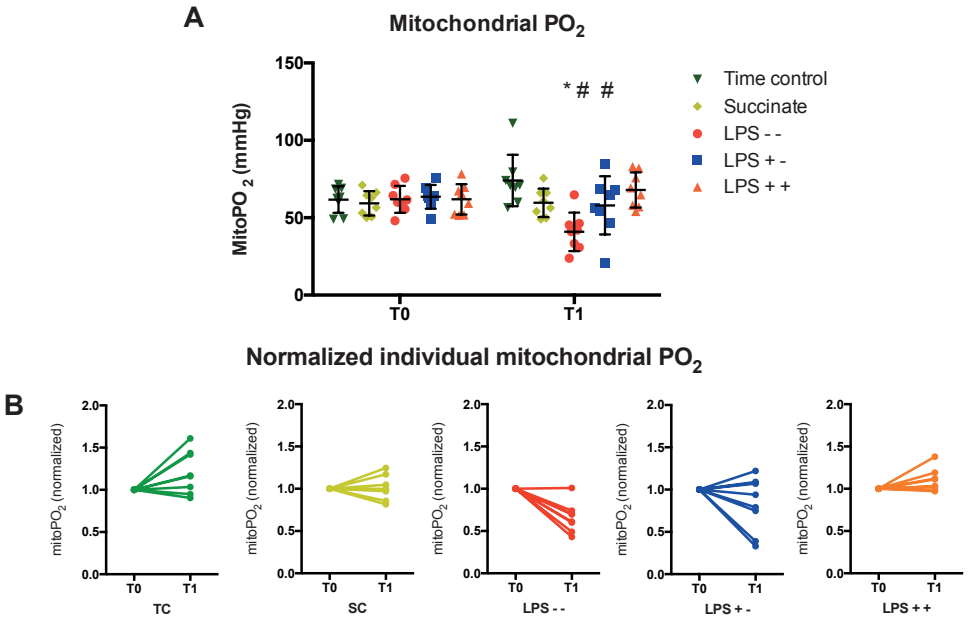

Figure 4. Panel A: $MitoPO_2$ at T0 and T1 in the different experimental groups. Panel B: Percentage change in individual $mitoPO_2$. TC; time control, SC; methyl-succinate control, LPS --; endotoxemia, LPS +-; endotoxemia with fluid resuscitation, LPS ++; endotoxemia with fluid resuscitation and methyl-succinate administration, LPS; lipopolysaccharide, $mitoPO_2$; mitochondrial oxygen tension. T0 and T1 are the time points of the $mitoPO_2$ measurement. Data are presented as mean ± SD, * significant difference compared to baseline measurement, # significant difference compared to time control measurement ($p<0.05$); (8 rats/group). LPS -- T1 vs LPS -- T0 ($p=0.0015$), LPS -- T1 vs TC T1 ($p<0.0001$) and LPS +- T1 vs TC T1 ($p=0.0006$).

ODR measurements

The ODR measurements at baseline (T0) did not differ significantly between the groups. In three groups ODR at T1 decreased compared to the baseline measurement (T0); the succinate control decreased from 4.6 ± 1.2 $mmHg*s^{-1}$ to 3.6 ± 0.5 $mmHg*s^{-1}$, the LPS -- group decreased from 4.5 ± 1.0 $mmHg*s^{-1}$ to 2.4 ± 1.6 $mmHg*s^{-1}$, and the LPS +- group decreased from 4.2 ± 1.3 $mmHg*s^{-1}$ to 3.1 ± 1.0 $mmHg*s^{-}$. However, infusion of methyl-succinate maintained ODR in the endotoxemia group (LPS++) at baseline values (Fig. 5).

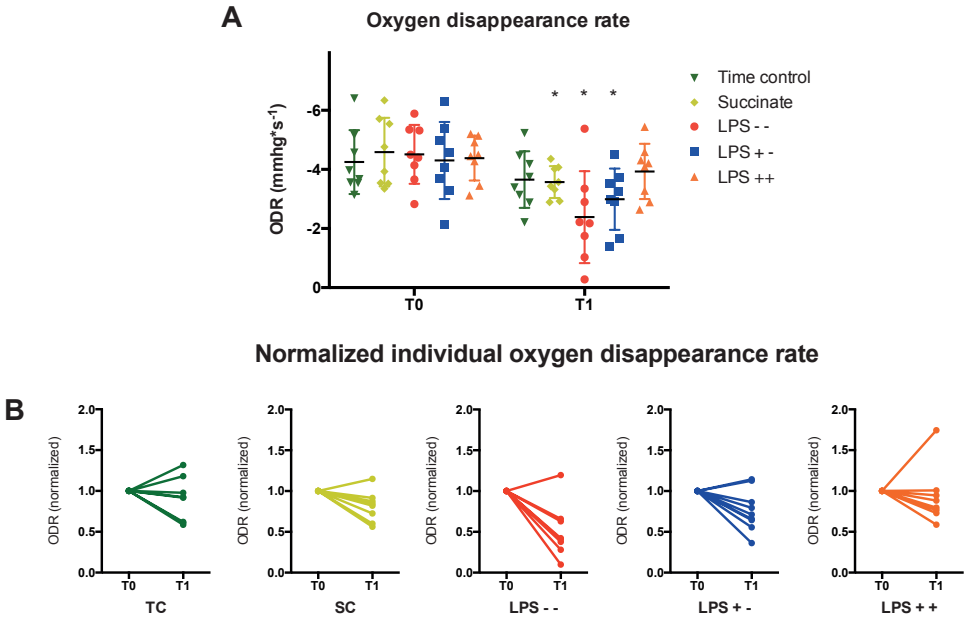

Figure 5. Panel A: ODR at T0 and T1 in the different experimental groups. Panel B: Percentage change in ODR TC; time control, SC; methyl-succinate control, LPS --; endotoxemia, LPS +-; endotoxemia with fluid resuscitation, LPS ++; endotoxemia with fluid resuscitation and methyl-succinate administration, LPS; lipopolysaccharide, ODR; mitochondrial oxygen disappearance rate. T0 and T1 are the time points of ODR measurement. Data are presented as mean ± SD, * significant difference compared to baseline measurement, # significant difference compared to time control measurement ($p<0.05$); (8 rats/group).intergroup analysis T1: SC vs TC ($p=0.034$), LPS -- vs TC ($p=0.0034$) and LPS+- vs TC ($p=0.0223$)

Discussion

The major findings of this study are that *in vivo* respirometry and *ex vivo* respirometry provide different results in our endotoxemic rat model and that PpIX-TSLT provides a sensitive means to measure aspects of mitochondrial function *in vivo*. LPS decreased *in vivo* muscle ODR at the measured timepoints compared to a time control group, but no effect of LPS was found in *ex vivo* mitochondrial function tests. No change in mitochondrial function was found using the high-resolution respirometer nor using direct complex activity measurements. In an LPS-induced rat endotoxemia model we showed a decrease in *in vivo* mitochondrial function (ODR) using the PpIX-TSL technique, that could be prevented by pre-treatment with methyl-succinate. This result confirms the previously measured beneficial effects of methyl-succinate in *ex vivo* biopsies from

endotoxemic rats (Protti et al.). In addition, it shows the feasibility of the PpIX-TSLT as an *in vivo* monitoring tool to determine the therapeutic effect of mitochondrial targeted drugs.

The administration of LPS leads to inhibition of complex I of the mitochondrial electron transport chain (Choumar et al., 2011), measured as a decrease of mitochondrial oxygen disappearance rate (Harms et al., 2015a). We did not replicate the detrimental effect of endotoxemia on *ex vivo* mitochondrial respiration experiments as shown by Protti et al. However, Protti et al used a different model (cegal ligation and puncture) to initiate an endotoxemia with no fluid resuscitation. Furthermore, their experiment lasted longer and they only used clinical severity grade as a marker for sepsis at 48 hours. This discrepancy with our results might imply that changes in ODR precede the changes measured with *ex vivo* mitochondrial function tests.

In the measurement of mitoPO$_2$ directly on the muscle a marked increase of mitoPO$_2$ on T1 was found compared to T0 in the time control group but not in the LPS treated group. In 6 hours a wound starts with it first phases of the healing process, it is unclear if a higher or lower oxygen concentration is expected since both are mentioned (Gottrup et al., 1984; Niinikoski et al., 1972). Endotoxemia has a marked diminutive effect on the pathophysiology of wound healing, this may explain the relatively low mitoPO$_2$ found in the LPS group compared to the time control group (Kawaguchi et al., 1995).

In our *in vivo* respirometry experiments, the administration of LPS resulted in decreased ODR. Fluid resuscitation in the LPS+- group prevented macro-hemodynamic deterioration, although a significant lower value of mitoPO$_2$ was found compared to time control, this may be in part explained by a relatively high mitoPO$_2$ found in the time control group at T1. A decrease in MAP and cardiac output often occurs after rapid and/or long-term LPS infusion (Harms et al., 2015a). In all our endotoxemia groups, lactate was significantly higher compared to the control groups. Lactate is only a crude marker of disease, depicting the balance between aerobic and aerobic metabolism in the tissues at the one site and on the other site an indication of slowing of liver metabolism. An increase of mitoPO$_2$ in the time control group, although not significant, may overestimate the changes found compared to the other experimental groups. The mitoPO$_2$ of 74 ± 17 mmHg at T1 in the time control group may be explained by a relative liberal fluid regime (succinate infusion was replaced with extra saline infusion in the other groups) used in this experiment. A constant mitoPO2 in the succinate control group indicates unaltered and adequate tissue oxygenation. Despite maintained mitoPO2 values at T1, the ODR of

the succinate control group, the LPS -- group and the LPS with fluid resuscitation group (LPS+-) showed a significant decrease compared to baseline measurements. Unclear is why succinate decreased ODR slightly compared to baseline, of interest is the decline of standard deviation compared to time control. Moreover, no intergroup differences were found on T1 in the ODR measurements. However, the greatest difference was observed between the LPS +- and the succinate and fluid resuscitated LPS-group (LPS++), in which ODR values did not decline from baseline values. Importantly, previous PpIX-TSLT measurements showed that mitochondrial respiration is independent of mitoPO_2 levels (Harms et al., 2013). Therefore, a distinction between problems related to oxygen supply or oxygen consumption on the cellular level in critical illness could be made using the PpIX-TSLT technique.

Several mitochondrial respiration studies have shown that mitochondrial oxygen consumption recovers after administration of succinate (Protti et al., 2007; Silva and Oliveira, 2011). Protection of ODR by methyl-succinate administration can be explained by several effects. Methyl-succinate could increase mitochondrial membrane permeability, increase substrates in the citric acid cycle, or increase the activity of complex II.

In our *in vivo* experiments, the infusion of methyl-succinate in the LPS-treated rats (LPS++) prevented a decline in ODR from baseline values. This demonstrates that *in vivo* ODR measurements are able to show changes in mitochondrial respiration which are subtler than changes in lactate. We reproduced the beneficial effect of methyl-succinate on mitochondrial oxygen consumption as previously found in isolated mitochondria (Protti et al.). We therefore think that our novel measurement method enables efficient monitoring of *in vivo* changes in mitochondrial respiration.

In our study, methyl-succinate was already administered before the LPS mediated endotoxemia was induced, which is not comparable to the clinical situation. Therefore, our data mainly demonstrates the feasibility of our experimental technique to detect subtle changes in mitochondrial respiration. Further research is needed to determine whether the protective effect of methyl-succinate is still present when administered after endotoxemia has already developed.

The clinical applicability of PpIX-TSLT (Harms et al., 2016; Mik, 2013) allows non-invasive real time monitoring of mitochondrial function. The absence of tissue damage and functional loss may overcome some of the current disadvantages experienced with biopsies (Jeger et al., 2013) and enables bedside monitoring. A limitation of the technique is the need to build up the PpIX signal following ALA application for at least 3 hours

(Harms et al., 2015b). The recent release of a monitor based on the PpIX-TSLT technique (Ubbink et al., 2016) provides a bedside clinical tool to monitor the deterioration and recovery of mitochondrial function in critically ill patients. Other potential clinical uses of PpIX-TSLT are related to resuscitation and blood transfusion management (Römers et al., 2016).

In conclusion, we showed the feasibility to monitor changes in mitochondrial respiration in endotoxemic rats by cutaneous PpIX-TSLT measurements. A decrease in ODR in the presence of preserved or restored mitoPO$_2$ suggests that mitochondrial dysfunction may be at the basis of the metabolic failure in sepsis, even in the absence of hemodynamic shock. The ability to measure mitochondrial ODR and mitoPO$_2$ in the clinical setting using bedside monitoring PpIX-TSLT is expected to contribute to a better understanding of mitochondrial dysfunction.

In addition, it will allow to monitor existing therapeutic approaches and develop improved therapeutic approaches aimed at restoring aerobic metabolism and cellular function.

List of abbreviations

ALA: 5-Aminolevulinic acid; LPS: Lipopolysaccharide; LPS-FR: Lipopolysaccharide with fluid resuscitation; LPS-LR: Lipopolysaccharide with late resuscitation; LPS-NR: Lipopolysaccharide without resuscitation; mitoPO2: Mitochondrial oxygen tension; ODR: Oxygen disappearance rate; PpIX: Protoporphyrin IX; PpIX-TSLT: Protoporphyrin IX-triplet state lifetime technique; RDM: Rectangular distribution method.

Competing interest

Dr. E.G. Mik is founder and shareholder of Photonics Healthcare B.V., Utrecht, The Netherlands. Photonics Healthcare B.V. holds the exclusive licenses to several patents regarding this technology, filed and owned by the Academic Medical Center in Amsterdam and the Erasmus Medical Center Rotterdam, the Netherlands.

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Chapter 6

Measuring mitochondrial oxygenation and respiration in vivo in a human endotoxemia model

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Abstract

In vivo measurement of mitochondrial respiration and acute changes thereof is notoriously difficult. We measured changes in mitochondrial oxygen tension (mitoPO$_2$) and mitochondrial oxygen consumption (mitoVO$_2$) using the bedside COMET (Cellular Oxygen METabolism) system during endotoxin-induced systemic inflammation in humans *in vivo*. 48 healthy male subjects received 2 ng/kg lipopolysaccharide (LPS) intravenously, without (LPS-group, n = 12) or with a cold- and or respiration-induced intervention (Intervention-group, n = 36). Four subjects, receiving no LPS, served as controls group. MitoPO$_2$ and mitoVO$_2$, were measured: just prior to LPS administration and 1.45 hours, 4 hours, and 7 hours thereafter, and at the corresponding timepoints in the control group. In the control group no significant changes over time in were found. COMET measurements were available in 10 of participants in the LPS group. MitoPO$_2$ decreased from 61[53-72] mmHg at baseline to 42[37-51] mmHg at 1.45 hours post-LPS ($p<0.01$) and returned to baseline afterwards. MitoVO$_2$ showed an increasing trend, without significant changes in the post-hoc analysis. Our results indicate that the COMET monitor can detect changes in mitochondrial parameters in a relatively mild model of systemic inflammation. This study paves the way for bedside monitoring of alterations in mitochondrial oxygenation and respiration.

Trial registration

ClinicalTrials.gov, NCT03240497.
toetsingonline.nl, NL65767.078.18

Key words: Mitochondria, Endotoxemia, Lipopolysaccharides, Healthy volunteers, 5-aminolevulinic acid,

Introduction

Mitochondrial function is of pivotal importance in cellular function. Subtle changes in mitochondrial function over time are thought to play a role in the development of several chronic diseases like Alzheimer's disease and type 2 diabetes[1]. Mitochondrial dysfunction is also implicated to play an important role during critical illness where oxygen demand, supply and consumption may be impaired. For example, in sepsis, where failure of microcirculation and a diminished mitochondrial function is related to development of multi-organ failure and death, irrespective of age [2,3]. Measuring the balance between supply and demand of cellular oxygen might aid clinical evaluation of sepsis or guide patient-based care protocols [4,5]. Direct, non-invasive measurement of the concentration of cellular oxygen and mitochondrial function in intact tissue reflects this balance between supply and demand of cellular oxygen levels[6]. Recently, the Cellular Oxygen METabolism (COMET) measuring system has been developed, which enables bedside measurement of mitochondrial oxygenation and respiration[7].

The COMET device measures cutaneous mitochondrial oxygen tension (mitoPO$_2$) over time. MitoPO$_2$ is measured by means of delayed fluorescence of mitochondrial protoporphyrin IX (PpIX)[8]. Microcirculatory flow can be stopped by applying pressure with the measuring probe on the skin, enabling the determination of the mitochondrial oxygen consumption (mitoVO$_2$) [9]. The mitoVO$_2$ measurement is a non-invasive technique to assess mitochondrial respiration *in vivo*. Using the COMET device, the technical feasibility to measure mitoPO$_2$ and mitoVO$_2$ in humans has been demonstrated in healthy volunteers [10]. Another research group recently showed little effect of physical activity on mitoPO$_2$ and mitoVO$_2$ measured with the COMET monitor [11].

In this study, we investigated the feasibility of the COMET system to detect changes in mitochondrial oxygenation and respiration during experimental human endotoxemia, a standardized well-controlled and reproducible model of systemic inflammation elicited by administration of *E. coli* lipopolysaccharide (LPS)[12]. We chose to use this model because previous work in rats has shown that LPS administration exerts detrimental effects on the mitochondrial oxygen consumption in skin, which was unrelated to alterations in mitochondrial oxygen tension[13].

Materials and Methods

Subjects and ethics

Data were collected from 52 healthy, nonsmoking, Dutch male volunteers of which 48 participated in an endotoxemia study registered at ClinicalTrials.gov as NCT03240497 and performed at the Radboud university medical center in Nijmegen, the Netherlands. The aim of the endotoxemia study was to investigate the effects of a training program consisting of a breathing exercise and/or exposure to cold on the inflammatory response induced by intravenous administration of LPS. A 2 by 2 design was employed in which 48 participants were randomized to 4 different groups (n=12 per group): cold exposure, breathing exercise, cold exposure and breathing exercise, and no training. All subjects in these 4 groups received LPS. Because especially the vigorous breathing exercise performed during the endotoxemia experiment may interfere with the mitochondrial measurements, only combined baseline mitoPO$_2$ and mitoVO$_2$ data (obtained before LPS administration) of the groups that were trained in cold exposure and/or the breathing exercise were used (LPS + intervention group, n=36). The untrained group is henceforth referred to as the LPS group (n=12). Details of the training procedures are described elsewhere [article in revision, attached with current submission]. Following completion of the endotoxemia study, we performed time-control experiments in 4 additional control subjects who received no LPS in a separate protocol (henceforth described as the control group). These experiments were conducted in the Erasmus Medical Center in Rotterdam, the Netherlands and registered at toetsingonline.nl as NL65767.078.18. Measurement timepoints were based on the changes in mitoPO$_2$ found in the LPS group (Baseline vs T1.45 hours after LPS). Both study protocols received institutional review board approval (CMO 2016-2312/ NL56686.091.16 and MEC 2018-090/NL65767.078.18). All subjects provided written informed consent and experiments were in accordance with the Declaration of Helsinki, including current revisions, and Good Clinical Practice guidelines. Subjects were screened before the start of the experiment and had a normal physical examination, electrocardiography, and routine laboratory values. Exclusion criteria were: febrile illness in the 2 weeks before the experiment, taking any prescription medication, history of spontaneous vagal collapse, participation in a drug trial or donation of blood 3 months prior to the experiment, porphyria, or participation in a previous trial where LPS was administered.

Study procedures

In both the endotoxemia and time-control protocols, subjects refrained from consuming caffeine or alcohol 24 hours before the start of the experiment, and from food 10 hours before the start of the experiment. An ALAcare 8mg plaster (Photonamic GmbH & Co KG, Wedel, Germany) was applied 8-10 hours before the first mitochondrial respiration measurement with the COMET (off-label use approved by the institutional review board). At the end of the experiment, the measurement site was covered by a plaster to prevent exposure to light for a day. The following morning, debriefing of the volunteers followed with attention to possible side effect of LPS and the plaster. A schematic overview of the procedures is provided in Fig. 1.

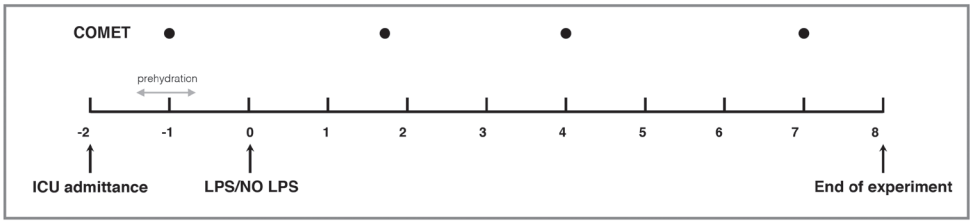

Fig. 1. Schematic overview of the experimental procedures. After fasting for 10 hours, subjects were prehydrated during 1 hour before administration of LPS (LPS group) or no administration (control group). Mitochondrial respiration and blood gas parameters were measured at 4 time points during the experiment. The x-axis represents hours relative to LPS/no LPS administration.

In the endotoxemia protocol, purified LPS (Clinical Center Reference Endotoxin derived from Escherichia coli O:113, obtained from Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD, USA)) supplied as a lyophilized powder, was reconstituted in 5 mL saline 0.9% for injection and vortex mixed for at least 20 min after reconstitution. The LPS solution was administered as an i.v. bolus injection at a dose of 2 ng/kg body weight in 1 min at T_0.

In both protocols, a cannula was placed in an antecubital vein to permit infusion of 2.5% glucose / 0.45% solution; all subjects received 1.5L 2.5% glucose / 0.45% saline during 1 hour, starting 1 hour before T_0 (prehydration) as part of the standard endotoxemia protocol [12], followed by 150 mL/h for 6 hours and 75ml/h until completion of the experiment. All the volunteers remained in bed during the entire experiment. Baseline COMET measurements were performed before the start of prehydration. Heart rate (three-lead electrocardiogram), blood pressure, and oxygen saturation (pulse oximetry)

data were recorded starting 1 hour before administration of LPS until discharge 8 hour post-LPS, and at corresponding timepoints in the control group. Body temperature was measured using an infrared tympanic thermometer (FirstTemp Genius 2; Sherwood Medical, Mansfield, UK). As described previously [12], headache, nausea, shivering, muscle and back pain were scored on a six-point Likert scale (0 = no symptoms, 5 = worst ever experienced), resulting in a total sickness score of 0–25. In the endotoxemia protocol, the radial artery was cannulated using a 20-gauge arterial catheter (Angiocath; Becton Dickinson, Sandy UT, USA) and connected to an arterial pressure monitoring set (Edwards Lifesciences, Irivine, CA, USA) to allow continuous monitoring of blood pressure and continuous blood sampling for blood gas analysis various timepoints (see Fig. 1). In the control group, blood pressure was measured noninvasively using a cuff.

Measurement of mitoPO2 and mitoVO2 using the COMET monitor

Oxygen-dependent quenching of the delayed fluorescence lifetime of mitochondrial PpIX is the first known method to measure mitoPO_2 in living cells and tissues, in a non-invasive and feasible manner in humans. PpIX is the final precursor of heme in the heme biosynthetic pathway. PpIX is synthesized in the mitochondria and administration of 5-aminolevulinc acid (ALA) substantially enhances the PpIX concentration. We used ALAcare plasters for PpIX enhancement. Photoexcitation of PpIX populates the first excited triplet state and causes the emission of red delayed fluorescence. The delayed fluorescence lifetime is inversely related to the mitoPO_2 according to the Stern–Volmer equation. The background of the delayed fluorescence lifetime technique is extensively described elsewhere[8,14–16].

The light source and the detection system are the two core components of the COMET monitor (Photonic Healthcare, Utrecht, the Netherlands). A 515 nm pulsed laser, pulse duration 60ns, with a 10Hz repetition rate illuminates the intra cellular accumulated PpIX. The fluorescent signal is projected on a gated red-sensitive photomultiplier tube. The light emitted by the sensor is divergent and safe for eyesight at any distance. A detailed description of the COMET measuring system can be found elsewhere[7].

Local oxygen consumption is measured as mitochondrial oxygen consumption (mitoVO_2), by pressure-induced occlusion of the microcirculation thereby stopping local oxygen supply. mitoVO_2 and mitoPO_2 were measured with the COMET at baseline, 1.45 hours, 4 hours and 7 hours post-LPS administration, and at corresponding timepoints

in the control group (Fig. 2A). These time points were chosen to match the start of the experiment (baseline), the peak of cytokine release (1.45 hours after LPS) the peak in temperature rise (4 hours after LPS) and the end of the experiment (7 hours after LPS). All COMET measurements were performed in the supine position. At baseline the volunteers were rested supine for at least 30 min after venous and arterial puncture to minimize the effect of stress on the baseline measurement. Measurement of the mitoPO$_2$ and mitoVO$_2$ were performed by the first author on both locations. The measurement probe was held above the ALA treated skin by hand. Occlusion of the microcirculation in the skin was achieved by manual firm pressure with the measurement probe (Fig. 2B). This simple procedure repeatedly created a measurable mitoVO$_2$, due to cessation of the microvascular oxygen supply and ongoing cellular oxygen consumption. The mitochondrial oxygen concentration was measured before and during application of pressure at an interval of 1 Hz, using two laser pulses per measurement. The mitoVO$_2$ is analyzed using Michaelis-Menten kinetics (Fig. 4). We previously described these principles in detail and provided a working implementation of the technique for mitoVO$_2$ measurements[16].

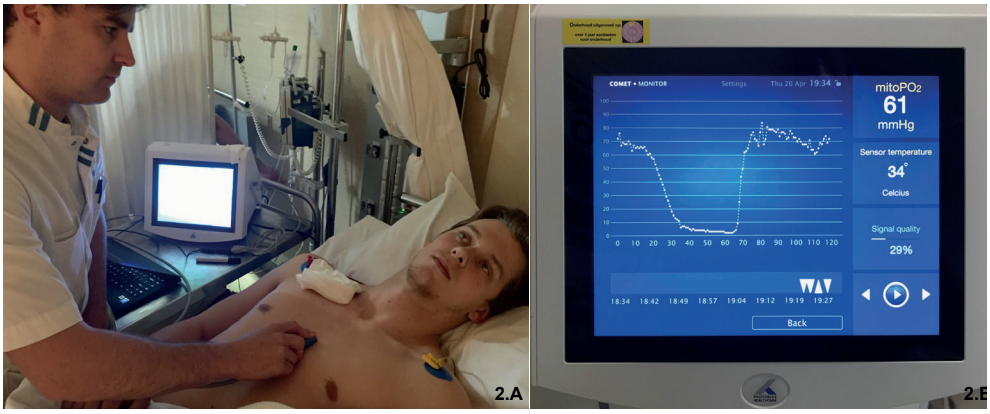

Fig 2. COMET measurements. A. Dynamic measurement with COMET setup in the experiment. Microcirculation of subject's skin was stopped by direct pressure with the measurement probe by the researcher. B. COMET monitor display showing an ongoing dynamic measurement of mitoVO$_2$. Raw data of COMET monitor was captured from the COM-port using a laptop. Photograph shown with permission of subject.

Statistics

Data are represented as mean and standard deviation or median with range/interquartile range based on their distribution (assessed using the D'Agostino & Pearson omnibus

test). To analyze differences over time within groups, Friedman's test were performed followed by Dunn's post hoc multiple comparison test vs. baseline measurements. The sample size of 4 in the time-control study was based on a power analysis using data from the LPS group: a mean change of 18.4 mmHg with a standard deviation of 11 mmHg, a significance level of 5% and a power of 80%. Two-sided p values of <0.05 were considered statistically significant. Statistical calculations were performed using Graphpad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

Results

Hemodynamic and clinical parameters

Baseline characteristics of the study subjects are listed in Table 1 and revealed no between-group differences. In the control group, no changes in heart rate, MAP, temperature or symptom score were observed during the experiment (Fig. 3). In the LPS group, a significant decrease in MAP (median [IQR]) from 98 [93-108] mmHg at baseline to 85 [80-94] mmHg at 4 hours and 83 [81-94] mmHg at 7 hours and post-LPS was observed (Fig. 3A). Heart rate (median [IQR]) showed a compensatory increase at the same timepoints: from 63 [60-75] at baseline to 96 [88-98] bpm at 4 hours and 88 [80-91] bpm at 7 hours post-LPS (Fig. 3B).

Table 1. Demographic characteristics.

	All participants (n=52)	**Control (n=4)**	**LPS (n=12)**	**LPS + intervention (n=36)**
Age, years	22 [20-24]	21 [14]	22 [20-22]	22 [20-24]
BMI, kg.m^{-2}	23.4 [22.2-24.6]	24.2 [6.2]	23·1 [22·2-23·9]	23.5 [22.2-24.6]
Systolic blood pressure, mmHg	139 [131-152]	121 [24]	137 [122-156]	140 [136-152
Diastolic blood pressure, mmHg	72 [65-79]	71 [8]	73 [66-82]	72 [64-79]
Heart rate, bpm	64 [56-71]	65 [21]	66 [59-75]	64 [55-67]

Data are presented as median and [range] for control group and median and [IQR] for all other groups. kg: kilogram, m: meter, bpm: beats per minute, mmHg; millimeters mercury.

Temperature (median [IQR]) increased from 36.8 [36.4-37.0]°C at baseline to 38.5 [38.2-38.8] °C at 4 hours after LPS administration, followed by a decrease to 37.8 [37.7-37.9] °C at 7 hours post-LPS (Fig. 3C). The total symptom score was 9 [3-11] (median [IQR]) points (out of a maximum of 25) at 1.45 hours after LPS administration (Fig. 3D) and remained elevated at 4 hours after LPS (6 [4-11]). No relevant changes in blood gas parameters were observed following LPS administration, although alterations in pH ,pCO_2, and HCO_3^- were statistically significant (Table 2).

Table 2. Arterial blood gas parameters during experimental endotoxemia in the LPS group

Time (hours relative to LPS)	**-1**	**0**	**1.5**	**3**	**p-value**
pH	7.40 [7.39-7.42]	7.38 [7.36-7.40] **	7.40 [7.39-7.42]	7.41 [7.39-7.42]	0.002
pCO_2 (mmHg)	39.7 [38.3-42.0]	39.6 [36.4-41.0]	38.0 [35.0-40.3]*	36.8 [33.3-40.0] *	0.02
PO_2 (mmHg)	114[109-118]	123[114-127]	117[103-129]	106[98.9-115]	0.16
HCO_3^- (mmol/l)	25.2 [23.8-26.2]	23.3 [21.8-24.2] **	23.0 [22.0-24.6] ***	23.7 [21.0-24.4] **	0.0006
sO_2 (%)	98[98-99]	99[98-99]	99[98-99]	98[98-99]	0.55
Lactate (mmol/l)	0.7[0.5-0.8]	1.0[0.9-1.2]	1.0[0.7-1.1]	0.6[0.5-0.7]	0.55

pCO_2: arterial carbon dioxide pressure, PO_2: arterial oxygen pressure, BE: base excess, HCO_3^-: bicarbonate concentration, sO_2: percentage of arterial hemoglobin oxygenation. Data presented as median [interquartile range] of 12 subjects. P-values were calculated using Friedman tests. * $P<0.05$ compared to baseline, ** $P<0.01$ compared to baseline, *** $P<0.001$ compared to baseline calculated using Dunn's post-hoc tests.

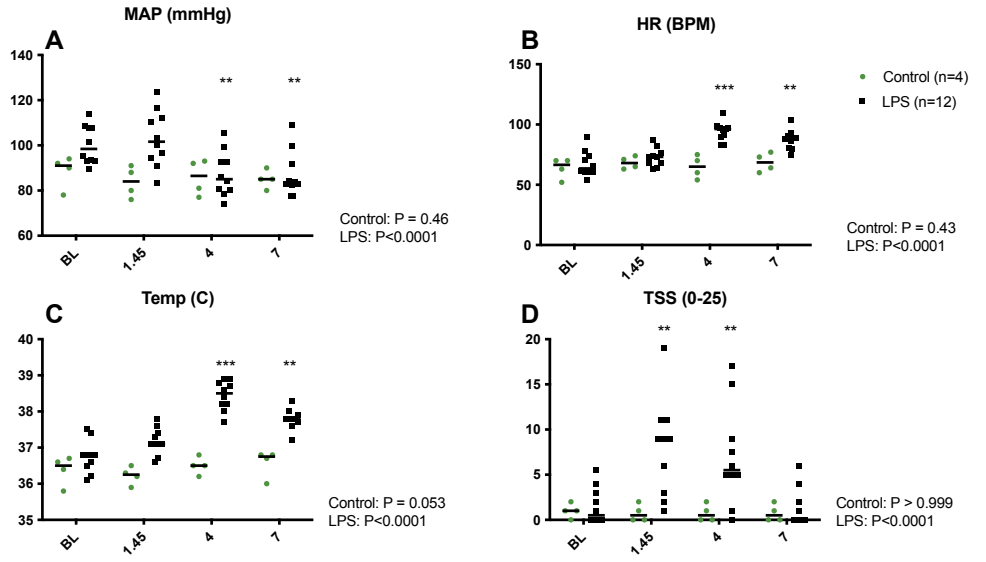

Fig 3. Measurement of vital parameters and symptom score over time. A. Mean arterial pressure (MAP, mmHg) B. Heart rate (HR, BPM) C. Temperature (Temp, C), Total symptom score (TSS, 0-25). Individual data and median values are shown. P-values in panels were calculated using Friedman tests. * P<0.05 compared to baseline, ** P<0.01 compared to baseline, calculated using Dunn's post-hoc multiple comparison tests.

Mitochondrial function parameters

At the debriefing, no side effects of the ALA plaster were observed or reported. Fig. 4 shows a representative example of the mitoVO$_2$ tracing for a single time point, analyzed using an adapted Michaelis-Menten fit procedure [16]. Analysis of the tracing with Michaelis-Menten kinetics yield an mitoVO$_2$ of -8 mmHg*s^{-1}, value for oxygen concentration change per second, and a baseline mitoPO$_2$ of 73 mmHg.

In 46 out of the total of 52 subjects in whom baseline measurements were performed, a sufficient PpIX signal was obtained (4 subjects did not follow instructions and applied the ALA-plaster later than told, 2 subjects showed an insufficient signal despite the fact that the plaster was timely applied). MitoPO$_2$ followed a normal distribution (D'Agostino & Pearson p-value of 0.41) with a mean and standard deviation of 61.8 ± 11.3 mmHg (Fig 5A). MitoVO$_2$ also followed a normal distribution (D'Agostino & Pearson p-value of 0.51) with a mean and standard deviation of -8.4 ± 1.7 mmHg*s^{-1} (Fig 5B).

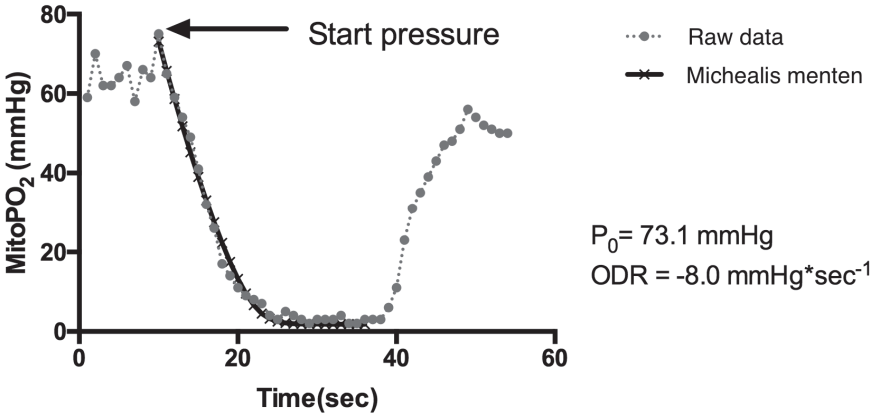

Fig 4. Michaelis Menten kinetics of mitochondrial oxygen concentration (mitoPO$_2$). Dynamic measurement of mitochondrial respiration, sampling rate of 1 Hz. Baseline measurement for 10 seconds where P$_0$ is determined. After a baseline measurement, local pressure by probe is applied which cuts oxygen supply. The cellular respiration causes the observed decrease in oxygen levels. At 38 seconds pressure is released and MitoPO$_2$ recovers.

In the control group 4 participants and in the LPS group 10 out of 12 participants gave sufficient PpIX signal. In the control group, no significant changes in mitoPO$_2$ or mitoVO$_2$ were observed throughout the experiment (Fig. 6A and B). In contrast, a significant decrease in mitoPO$_2$ (median [IQR]) was observed 1.45 hours after LPS administration compared with baseline (61 [53-72] vs. 41.7 [37-51] mmHg, Fig. 6A).

Although LPS administration did result in significant changes in mitoVO$_2$ over time (indicated by a Friedman test p-value <0.05), post-hoc tests did not reveal statistically significant differences on individual timepoints post-LPS compared with baseline, although an increasing trend was observed at later timepoints (Fig. 6B).

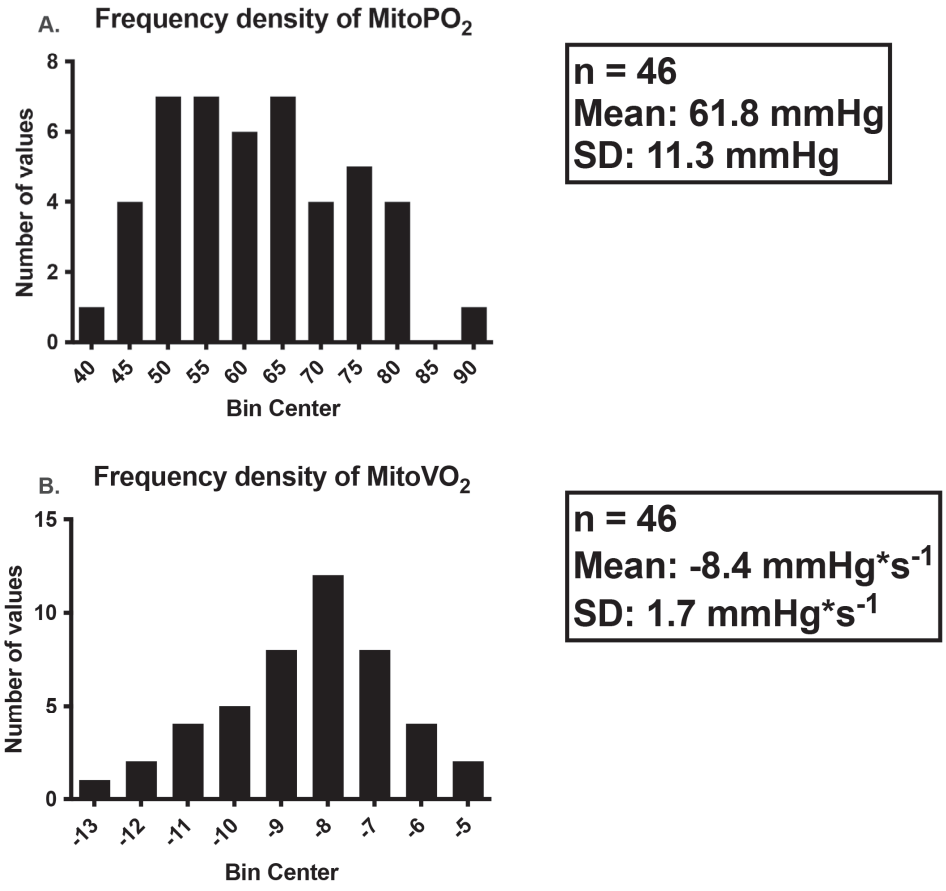

Fig 5. Histogram of baseline mitochondrial oxygen concentration (MitoPO2, panel A) and dynamic mitochondrial oxygen consumption (mitoVO$_2$, panel B). Data of all volunteers who had a sufficient PpIX signal (n=46) are depicted.

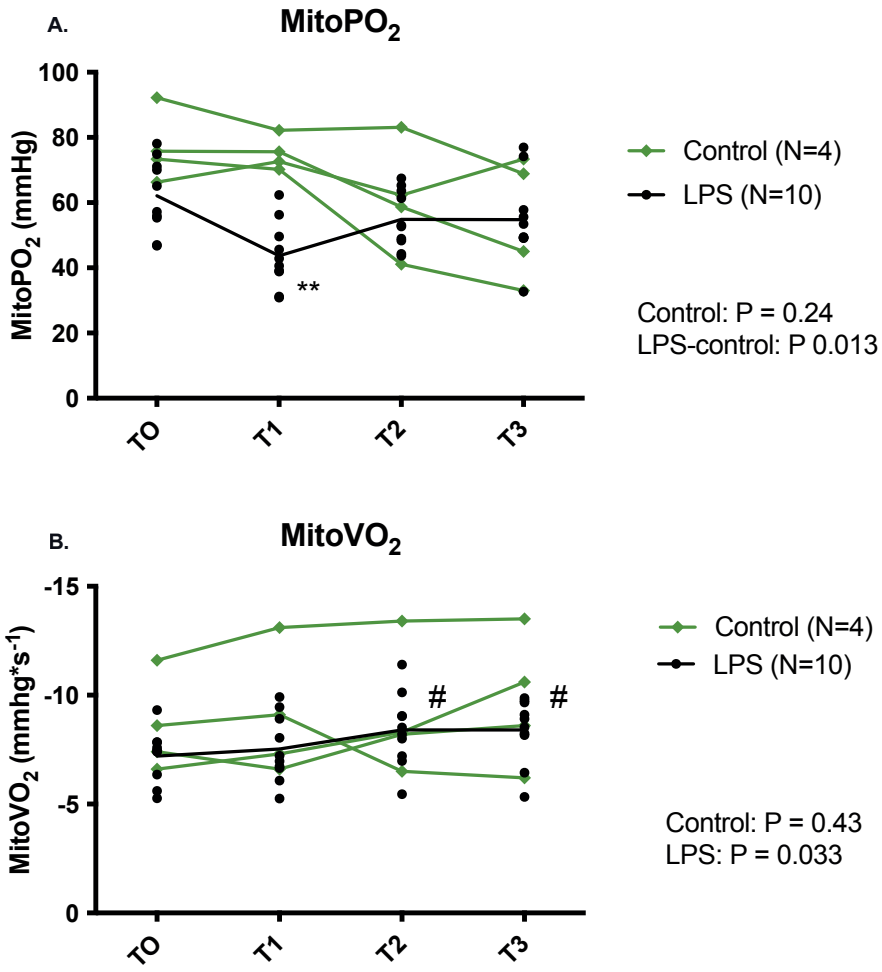

Fig 6. Mitochondrial oxygen parameters over time. A. Mitochondrial oxygen concentration (mitoPO$_2$). B. Dynamic mitochondrial oxygen consumption (mitoVO$_2$). Individual curves (control group) and individual data and median values (LPS group) are shown. P-values in panels were calculated using Friedman tests. # P=0.05-0.1 compared with baseline, ** P<0.01 compared with baseline, calculated using Dunn's post-hoc multiple comparison tests.

Discussion

In the present study, we were able to obtain mitoPO$_2$ and mitoVO$_2$ in 46 out of 48 subjects and the systemic inflammatory response induced by LPS administration results in decreased mitoPO$_2$ values and a trend towards increased mitoVO$_2$ levels. We demonstrate the feasibility of using the COMET measurement system to perform sequential mitochondrial respiration (mitoPO$_2$ and mitoVO$_2$) measurements in a dynamic setting during experimental human endotoxemia.

Due to the set-up of the experiments (volunteers arrived at the hospital early in the morning), we choose to apply the ALA-plaster longer before the first measurement than in earlier studies. In 46 out of 48 subjects who complied with the instructions pertaining to the application of the ALA plaster, a reliable PpIX signal was nevertheless obtained. The 4 subjects who did not comply with the instruction applied the plaster a maximum of 3 hours before the first measurement, so a relevant rise in PpIX was not expected.

The normal values measured by the COMET monitor determined in this study are higher compared to earlier work by our lab, however these previous measurements were performed using a preclinical monitor with a different probe[10]. Compared to COMET normal values reported in a previous exercise study performed by another group [11], mitoPO$_2$ values in the present work show a similar distribution. However, direct comparison of mitoVO$_2$ values between our study and theirs is difficult due to a different kind of mitoVO$_2$ analysis used, namely a mathematical approach [11] instead of our kinetic approach. A recently published verification of mitoPO$_2$ calibration in healthy volunteers showed accuracy of mitoPO$_2$ and thereby mitoVO$_2$ values[17].

To the best of our knowledge, we are the first to show changes in *in vivo* mitochondrial function during experimental human endotoxemia. LPS administration resulted in an early and significant decrease in mitoPO$_2$ and a nonsignificant and later-occurring increase in mitoVO$_2$. No changes were observed in the control group, indicating that these effects can be attributed to the LPS-induced systemic inflammatory response and not to diurnal variation. Similar to the present work, our previous studies in rats showed stable values of mitoPO$_2$ and mitoVO$_2$ in the control groups over time, and a sharp decline in mitoPO$_2$ following LPS administration, an effect that was reversed by aggressive fluid resuscitation [13,18] However, LPS administration decreased mitoVO$_2$ by 30% in rats [13,14]. This discrepancy with the results of the current study may be explained by the use of a high dosage of LPS in rats, resulting in a more severe grade of systemic inflammation [13,18].

In any case, the findings of this study signify that the COMET measurement system is also able to detect changes in a relatively mild model of systemic inflammation.

MitoPO_2 represents a measure of the balance between oxygen demand and supply in tissue. A decrease in mitoPO_2 may indicate a decrease in oxygen supply to the measurement site or an increase of oxygen usage while oxygen supply is maintained. Combined with the relatively minor change in mitoVO_2, the increased mitoVO_2 observed after LPS administration in the present study is more likely indicative of decreased oxygen supply and thus blood supply to the skin, marking the first step of centralization of blood supply during endotoxemia. Although lactate is frequently used as a marker of organ hypoperfusion in clinical practice, this study shows that mitoPO_2 may represent a more sensitive measure, as lactate did not change following LPS administration. A more pronounced change in mitoPO_2 compared to lactate shows promise for the skin as a primary target for measuring changes in oxygen supply on a cellular level as an early marker of circulatory impairment. Mismatch of local tissue perfusion and macrocirculation may cause an increase of lactate, but aggressive fluid loading did not change lactate concentrations[19]. As such, lactate may be associated with tissue hypoperfusion, but a direct correlation is questionable. A parameter that truly reflects local tissue perfusion and function might benefit the patient in guiding goal directed therapy. The detrimental effect of sepsis on mitochondrial function were already shown in thrombocytes obtained from adult and pediatric patients [3,20], showing an association between early mitochondrial function changes and development of multi-organ failure. Noninvasive measurement of mitochondrial function *in vivo* might be the step necessary for early diagnosis of mitochondrial dysfunction and stratification of patients in the intensive care unit[4].

Mitochondrial dysfunction is suggested to play a major role in the development of organ failure in sepsis[21]. Although the presences of pathophysiological changes in mitochondrial function in sepsis are well-described[22], the role of mitochondrial dysfunction in sepsis remains controversial[21]. Changes in mitochondrial function in blood cells, platelets and peripheral mononuclear blood cells, appear to be predictors of mortality and morbidity in adult and pediatric sepsis patients[3,23]. An *in vivo* bedside monitor could be of value for monitoring of mitochondrial dysfunction. Although we observed a trend towards increased mitoVO_2 several hours following LPS administration, it is important to stress that clinically relevant mitochondrial dysfunction was not to be expected in this relatively mild model of systemic inflammation. Furthermore, mitoVO_2 is a parameter of mitochondrial respiration; as such, a change in mitoVO_2 cannot be

interpreted as mitochondrial dysfunction per se. An increase of mitoVO$_2$ can be caused by an increase of ATP production, but may also be a sign of mitochondrial uncoupling during which mitochondrial oxygen usage is not coupled to ATP production. The observed trend towards a relatively late increase several hours following induction of systemic inflammation warrants a study in which daily measurements are made in patients admitted to the ICU with sepsis. In this respect, the COMET monitor could represent an important tool, offering frequent mitochondrial function monitoring during the day.

The translation of these findings to clinical sepsis are restricted for several reasons. We studied healthy volunteers between 20-35 years of age, while most sepsis patients are much older, with a recent sepsis study reporting nearly a quarter of the study population being above 75 years of age[24]. Moreover, the inflammatory stimulus in the model we used is relatively mild and only short-lived, as opposed to an ongoing and often severe infection in patients. Nevertheless, experimental human endotoxemia is a highly controlled and reproducible model used to investigate the inflammatory response and possible therapeutics in sepsis[25–27]. For instance, the human efficacy of biologics as anakinra and infiliximab, were first established in the experimental human endotoxemia model[28,29].

Conclusion

We show feasibility of measuring kinetics of mitochondrial oxygen tension and cellular oxygen usage in a model of systemic inflammation in humans *in vivo*, using a method that was previously established in rats[9,13,18]. We demonstrate a significant decrease in mitoPO$_2$ and a nonsignificant increase in mitoVO$_2$ following LPS administration. These findings show technical feasibility of more than once daily measurements of mitochondrial function parameters with the new COMET monitor, paving the way for clinical implementation of *in vivo* bedside monitoring of mitochondrial respiration.

Key messages

frequent monitoring of mitoPO$_2$ and mitoVO$_2$ during the day in humans is possible using the noninvasive COMET measurement system.

The COMET measurement system can detect changes in mitoPO$_2$ and, to a lesser extent, mitoVO$_2$ in a relatively mild model of systemic inflammation in humans.

List of abbreviations

COMET: Cellular oxygen metabolism; ALA: 5-Aminolevulinic acid; LPS: Lipopolysaccharide; mitoPO$_2$: Mitochondrial oxygen tension; mitoVO$_2$: Mitochondrial oxygen consumption; PpIX: Protoporphyrin IX; MAP: mean arterial pressure

Declarations

Ethics approval and consent to participate

The study protocol was approved by the local ethics committee of the Radboud University Nijmegen Medical Centre (CMO 2016-2312) and the Erasmus University Hospital (MEC 2018-090)

Consent for publication

Participant gave consent to publication for data and images shown.

Availability of data and material

The dataset generated and/or analyzed during the current study is not publicly available due to subject-related confidentiality, but is available from the corresponding author on reasonable request.

Competing interest

Dr. E.G. Mik is founder and shareholder of Photonics Healthcare B.V., The company that developed and commercializes the COMET monitor. Photonics Healthcare B.V. holds the exclusive licenses to several patents regarding this technology, filed and owned by the Academic Medical Center in Amsterdam and the Erasmus University Medical Center Rotterdam, the Netherlands. The remaining authors declare that they have no competing interests.

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Authors contribution

MAWB performed experiments, analyzed data, and wrote the manuscript. JZ, PP, and MK designed the study, revised the manuscript critically, and edited the manuscript. MK conceived and supervised the study. BS performed experiments, analyzed data and revised the manuscript critically. EGM supervised the dynamic measurements, revised the manuscript critically, and edited the manuscript. All authors read and approved the final manuscript.

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Chapter 7

Interim analysis; Feasibility study of measurement of mitochondrial function in patients with sepsis

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INTRODUCTION

Sepsis remains a life-threatening disease with mortality rates ranging between 20-40%.(1, 2) The exact role of mitochondrial function in sepsis is unclear, but mitochondrial dysfunction may play a central role in its pathophysiology.(3–6) Mitochondrial dysfunction or elevated mitochondrial damage proteins in serum are linked to mortality in sepsis patients on the ICU.(7–9) Translating these insights from animal experiments to human studies or clinical practice has been hampered by technical difficulties and limitations. Measuring the mitochondrial function with *ex vivo* techniques, in isolated mitochondria or in small tissue biopsies, may not reflect the *in vivo* situation correctly.(10) Thus, showing feasibility of a non-invasive *in vivo* measurement technique to monitor mitochondrial (dys)function in critical illness would be of considerable value.

The recent development of the COMET monitor, a clinical monitor for assessment of Cellular Oxygen METabolism, allows cutaneous mitochondrial oxygen tension (mitoPO$_2$) and mitochondrial oxygen consumption (mitoVO$_2$) measurement in humans. COMET measures mitoPO$_2$ by means of the Protoporphyrin IX-Triplet State Lifetime Technique (PpIX-TSLT). The background of this technique was first published in 2006 by Mik et al.(11) Broadly, COMET measures mitoPO$_2$ levels through lifetime measurement of oxygen-dependent endogenous Protoporphyrin IX (PpIX). By stopping microvascular flow, oxygen transport to the mitochondria is disrupted and by tracking mitoPO$_2$ over time, mitoVO$_2$ can be determined. Cutaneous application of 5-aminolevulinic acid (ALA) is a necessary step to induce sufficient mitochondrial PpIX for detection of the weak delayed fluorescence signal.(11, 12) Since the first publication of this technique, extensive research has been done to develop an instrument for clinical use. (13) The technique has been tested and calibrated for use in isolated organs and *in vivo* in experimental animals, and positive results of a feasibility test in healthy volunteers have been published.(14)

COMET was able to detect meaningful changes in mitoPO$_2$ and mitoVO$_2$ concentrations *in vivo* in sepsis-like conditions in a rat model.(15, 16) The aim of this study was to replicate data of the experimental setup in patients, to validate the hypothesis that patients with sepsis will suffer from a decline of mitoVO$_2$ as compared to a control group. Recent validation of the COMET technique in healthy volunteers, combined with its low risk profile for determining *in vivo* mitochondrial function, paves the way for an observational study on the intensive care ward. However, an important outstanding question for use of the COMET technique on the ICU ward remains its reliability in patients with sepsis, since mitochondrial function is a prerequisite for the conversion of ALA to PpIX.

The most used *ex vivo* technique to measure mitochondrial oxygen metabolism is an high resolution respirometer.(17) However tissue biopsies are invasive and not feasible in ICU patients and is not performed in clinical practice. Recently a strong link was shown with mortality in sepsis patients and mitochondrial dysfunction measured in platelets. (18, 19) Furthermore measuring a high concentration of mitochondrial DNA in serum, only present if mitochondrial damage occurs, is predictive for mortality.(8, 9) Measuring *in vivo* mitochondrial dysfunction in ICU patients with the COMET monitor when *ex vivo* tests show altered mitochondrial function or damage could provide the first signal for a higher risk for mortality in patients at bedside.

The primary goal of this study was to evaluate the ability of the COMET monitor to measure mitoPO$_2$ and mitoVO$_2$ concentrations reliably in ICU patients. No prior data was available on the use of the COMET monitor in a real-life ICU setting. Secondary goals of this pilot study were to determine changes in mitoPO$_2$ and mitoVO$_2$ concentrations in sepsis patients on the first day of admission compared to measurements in ICU-admitted control patients. Moreover, COMET's measurements were compared to mitochondrial respiration in platelets and the concentration of mitochondrial DNA as measured in plasma.

Materials and methods

Subjects

For this study, adult patients between 18 and 70 years of age were recruited from the intensive care unit (ICU; maximum capacity of 36 staffed beds) of the Erasmus MC University Medical Center Rotterdam, which is the largest tertiary hospital in The Netherlands. Every morning all newly ICU-admitted patients were screened by the researcher or ICU research nurse. The study protocols received institutional research board approval (MEC-2016-540). The protocol was registered at toetsingonline.nl as NL65767.078.18. If possible, written and signed informed consent was obtained from the patient before the beginning of the study. Due to the ICU setting, even patients who were able to communicate, were often unable to determine for themselves if they were willing and able to participate in this study. Even if legal representatives are available at the time of inclusion, an informed consent conversation could be an unsolicited and avoidable burden at this point. However, the aim of this study was to measure mitochondrial changes in an developing sepsis and therefore created a need to prepare the measurement as soon as possible. So, considering the minimal risk of this study,

the MEC allowed for a deferred consent procedure. Deferred consent or deferred proxy consent had to be obtained within a maximum of 72 hours, and before preparation of the second measurement was started. If the patient or legal representatives objected, the subject was excluded from the study and data obtained from the first measurement was not used for further analysis and deleted. During the deferred consent process, some patients would be prepared for a first measurement, before informed (proxy) refusal was given.

The procedures were in accordance with the Declaration of Helsinki, including current revisions, and Good Clinical Practice guidelines. Time of admission allowed the first measurement to be taken in less than 24 hours after admission to the ICU. Admission to another centers' ICU, the post anesthesia care unit (PACU) or coronary care unit (CCU) prior to the current ICU-admission were considered as ICU time as well.

Exclusion criteria were: diagnosis of mental disability, diagnosis of mitochondrial disease, pregnancy or porphyria.

This study was a single-center prospective observational study in which we measured mitochondrial function parameters oxygen tension (mitoPO$_2$) and oxygen consumption (mitoVO$_2$) within 24 hours of admission to the ICU. Blood samples were taken directly after the oxygen consumption measurement with the COMET monitor. If patients were available a second measurement was performed 5-7 days after ICU admittance.

Patients with A) a positive qSOFA (quick Sepsis Related Organ Failure Assessment) score (≥ 2 criteria) and suspected infection; or B) a recent sepsis diagnosis by an intensivist or referring specialist, were included in the sepsis group. If patients did not have a positive qSOFA or a diagnosis of sepsis they were included in the control group. A standardized form was filled in at admission for each included patient with data from the medical record.

Study procedures

Measurement of mitoPO2 and mitoVO2 using the COMET monitor

Oxygen-dependent quenching of the delayed fluorescence lifetime of mitochondrial PpIX is the first known method to measure mitoPO$_2$ in living cells and tissues, in a non-invasive and feasible manner in humans. PpIX is the final precursor of heme in the heme

biosynthetic pathway. PpIX is synthesized in the mitochondria and administration of 5-aminolevulinc acid (ALA) substantially enhances the PpIX concentration. We used 8 mg ALAcare® plasters (photonamic GmbH und Co. KG, Pinneberg, Germany) for PpIX enhancement. Photoexcitation of PpIX populates the first excited triplet state, and causes the emission of red delayed fluorescence. The delayed fluorescence lifetime is inversely related to the mitoPO$_2$ according to the Stern–Volmer equation. The background of the delayed fluorescence lifetime technique is extensively described elsewhere.(20–22)

The light source and the detection system are the two core components of the COMET monitor (Photonic Healthcare, Utrecht, the Netherlands). A 515 nm pulsed laser, pulse duration 60ns, with a 10Hz repetition rate illuminates the intracellular accumulated PpIX. The fluorescent signal is projected on a gated red-sensitive photomultiplier tube. The light emitted by the sensor is divergent and safe for eyesight at any distance. A detailed description of the COMET measuring system can be found elsewhere. (23)

Mitochondrial oxygen consumption (mitoVO$_2$) is measured by pressure-induced occlusion of the microcirculation thereby stopping local oxygen supply. The measurement probe was held above the ALA treated skin by hand. Occlusion of the microcirculation in the skin was achieved by manual firm pressure with the measurement probe. This simple procedure reliably created a measurable mitoVO$_2$, due to cessation of the microvascular oxygen supply and ongoing cellular oxygen consumption. The mitochondrial oxygen concentration was measured before and during application of pressure at an interval of 1 Hz, using two laser pulses per measurement. The mitoVO$_2$ is analyzed using Michaelis-Menten kinetics. We previously described these principles in detail and provided a working implementation of the technique for mitoVO$_2$ measurements.

Platelet Mitochondrial Oxygen Consumption

Chemicals and Sample Preparation

All used chemicals were purchased from Merck, at the time of the order still known as Sigma-Aldrich (St Louis, MO, USA). In ICU-admitted participants, blood samples of about 10 mL were taken directly after completion of the COMET measurement from an existing arterial or venous line in K$_2$E (EDTA) 6.0 mL tubes (BD Vacutainer®, Becton Dickinson, Plymouth, United Kingdom). In patients without an existing arterial or venous line, for example after being transferred to other departments, blood was drawn via venous puncture in K$_2$E (EDTA) tubes right after the measurement. Platelet-rich plasma (PRP) was then prepared within 10 – 30 minutes after the blood sample was taken, by centrifugation

of the platelets for 15 minutes at 150 × *g* at room temperature (21 – 24 °C). 1.0 mL of this PRP was collected.

Previously published methods for measurement of mitochondrial respiration in platelets were followed.(18, 19, 24) Compared to the described protocols we did not see the benefit of first creating a platelet pellet and resuspending it in the same plasma. Therefore, we altered the platelet isolation protocol used in these studies. Hereby creating a PRP instead of a near protein free plasma and platelet pellet and 1 ml of PRP was transferred to a separate tube. The residue (hematocrit plus PRP) was centrifuged a second time for 6 minutes at 4,000 × *g* at room temperature (21 – 24 °C) and all plasma was collected. For the determination of free mitochondrial DNA in plasma as marker of mitochondrial damage(25), 200 µL of plasma was transferred to a 1.5 mL sterile tube (Sterile Safe-Lock™, Eppendorf®, Hamburg, Germany) and snap frozen in liquid nitrogen and stored at -80°C.

High-Resolution Respirometry (HRR)

Measurement of oxygen consumption in intact thrombocytes' mitochondria was performed in a high-resolution oxygraph (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Volume calibration was performed at the start of each week. Air calibration was performed on the air-saturated plasma of the patient at the start of each experiment. The oxygen solubility factor was set to 0.89 for plasma, the stirrer speed was set to 750 rpm and chamber temperature was set to 37□. . Data was recorded with DatLab 5.2 software (Oroboros Instruments, Innsbruck, Austria) with sampling rate set to 2 seconds.

The 2 mL glass chamber was filled with 1.6 mL of the air-saturated collected plasma of the patient, after which the oxygen calibration was performed. Previous published data suggested that a concentration of 100-200 × 10^6 platelets per milliliter in the oxygraph chamber yields superior results in respiration measures.(26) 0.5 mL of PRP was added to the plasma of both chambers.

If necessary, reoxygenation was performed until an adequate oxygen concentration was reached. After 10 – 15 minutes of stabilization, the unstimulated respiration state (state 4o) was reached. Oligomycin (4 µL, 5 mM) – an ATP synthase inhibitor – was added to induce an ADP phosphorylation independent respiration state in 15 – 30 minutes. The oxidative phosphorylation uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (20 mM) was repeatedly added in volumes of 1 µL, until no further increase in respiration was detected in two successive additions. 1 µL of rotenone and 2 µL of antimycin A were added, resulting in a remaining oxygen respiration primarily

attributed to non-mitochondrial oxygen consumption (proteins and enzymes in the serum). As these measurements were performed in serum rather than buffer, oxygen consumption of other processes in the platelets as well as oxygen consumption of the serum are part of the measured respiration. To correct for these facts, the remaining oxygen respiration rate was subtracted from the measured unstimulated respiration rate, the ADP phosphorylation independent respiration state and the maximal respiration rate. Figure 1 shows a representative oxygen respiration curve measured with HRR. The final platelet concentration in the chamber was measured at the Department of Clinical Chemistry (AKC) using an automated hematology analyzer (XN-10, Sysmex®, Kobe, Japan). The O_2 flux per volume was then corrected for platelet concentration.

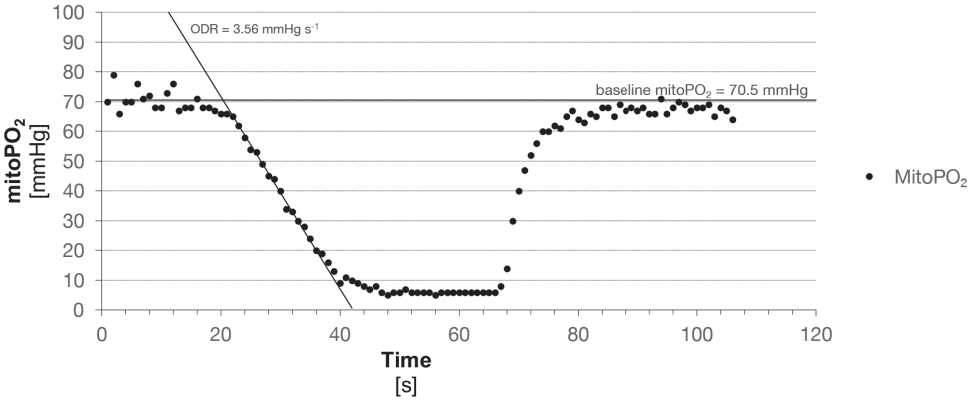

Figure 1. Respirometry graph of oxygen consumption in platelets. Oxygen concentration per volume [nmol/mL] (Y_1) and flux per volume [pmol/(s × ml)] (Y_2) over time. Additions: OM (oligomycin) 4 µL, FCCP (11 times) 1 µL, R (rotenone) 1 µL and AM (antimycin A) 2 µL.

Mitochondrial DNA measurement

The analysis is based on the method described by Nakahira et al.(8) A short description follows.

DNA isolation from plasma

For DNA isolation the DNeasy Blood & Tissue kit from Qiagen was used (#69504, Qiagen, Hilden, Germany). Prior to DNA isolation the plasma was thawed on ice. 100 µl of plasma was diluted with 100 µl PBS. The samples were mixed using a vortex and centrifuged at 700xg for 5 minutes at 4°C. 190 µl of the supernatant was transferred to a new

Eppendorf tube followed by another centrifuge step at 16.000xg for 15 minutes at 4°C. A final volume of 170 µl of the supernatant was transferred to a new Eppendorf tube and was used for DNA isolation.

30 µl of PBS, 20 µl Proteinase K and 200 µl AL buffer (from DNA isolation kit) were added to the 170 µl supernatant. After mixing, the samples were incubated at 56°C for 15 min. After incubation 200 µl of absolute ethanol was added and mixed using vortex. The samples were then transferred to a DNeasy isolation column from the kit and the kit protocol was followed. In the final step the DNA was eluted in 200 µl of eluent (AE buffer).

qPCR

To analyse the levels of mitoDNA in the isolated DNA samples from plasma, the following primers were used: human NADH Dehydrogenase 1 (mtND1), forward primer: 5'-ATACCCATGGCCAACCTCCT-3', reversed primer: 5'-GGGCCTTTGCGTAGTTGTAT-3'. As a control for nuclear DNA the following primers were used: human β-globin, forward primer 5'- GTGCATCTGACTCCTGAGGAGA -3', reversed primer 5'-CCTTGATACCAACCTGCCCAG-3'. A final primer concentration of 400 nM was used. To quantify the levels of mitoDNA (mtND1) a gBlock gene fragment (synthetic dsDNA fragment of the mtND1 gene, made by IDT-DNA) was used as positive control. The gBlock gene fragments are provided dry and must be resuspended in IDTE to a final concentration of 10 ng/µl according to company instructions. The copy number/µl was calculated using the following formula:

C x M x (1x10-15 mol/fmol) x Avogrado's number = copynumber/µl

Where C = current concentration in ng/µl (=10 ng/µl), M= molecular weight in fmol/ng (= 5,22 according to datasheet delivered with the gBlock), Avogrado's number = 6,022 x 10^{23}. The calculated copy number/µl in the stock solution is 3,14 x 10^{10} copies/µl . A 10 fold dilution series from 3,14 x 10^{5} to 3.14 x 10^{0} was used as a standard.

To perform the qPCR analysis the SensiMix SYBR & Fluorescein kit, Bioline #QT615-05 (Meridian bioscience inc, Cincinnati, Ohio, USA) was used in combination with the Bio-Rad CFX96 real time system. The qPCR program was as follows: To start 2 min 50°C and 10 min 95°C, then 40 cycles of 15 sec 95°C & 1 min **58**°C. At the end a melting curve analysis was performed to check amplification specificity.

The data was analyzed using the qPCR software (Bio-Rad CFX manager 3.1

All samples and standards were measured in duplicates and a no template control

(negative control) was included. To prevent high Cq values (high Cq values means less accuracy) the DNA samples were used undiluted.

For converting the copies/µl DNA sample to copies/µl plasma the following formula was used:

c= Q x vF x Vdna / Vplasma

where **c** = copies/µl plasma, **Q** = copies/µl DNA calculated by qPCR software, **vF** = dilution factor (undiluted = 1), **Vdna** = Volume of extracted DNA (final step DNA isolation) (200 µl), **Vplasma** = Volume plasma used for DNA isolation (100 µl)

Statistical analysis

Descriptive analyses were used to report on baseline characteristics of both groups. For non-normal distributed variables medians and interquartile range (IQR) are shown. For dichotomous or categorical data, percentages are shown. All statistical analyses and figures were performed using GraphPad Prism 7 (La Jolla, California, USA).

RESULTS

Patients were screened between October 2017 and September 2019. We started the informed consent procedure with 24 patients. Of these eligible patients due to refusal of consent by proxy two control patients and two sepsis patient were not measured. We were not able to obtain final informed consent of four control patients and one sepsis patient. In the control group one measurement of the COMET failed probably due to a problem in the preparation phase. The measurement after 5-7 days were conducted in six patients of the control group and for one patient in the sepsis group.

During ICU admittance lactate measurements are 1.2 [0.7;1.4] mmol/liter in the control group and 1.5 [1.2;4.5] mmol/liter in the sepsis group. In our study groups 28 day mortality is 3/6 in the control group and 4/7 in the sepsis group. Baseline characteristics of all patients who underwent at least one measurement are described in Table 1.

Table 1. Baseline Patient Characteristics.

Variable	Controls (*n* = 6)*	Sepsis (*n* = 7)*
Age, mean ± SD (y)	64.9 [60 ; 66.2]	63.5 [58.0 ; 65.3]
Sex, n (%)		
Male	5 (83)	3 (43)
Female	1 (17)	4 (57)
ICU stay (d)	21.1 [18.7 ; 22.8]	7.4 [1.5 ; 24.2]
Comorbidities, n (%)	1 (17)	6 (85)
Hypertension	1 (17)	2 (29)
Coronary artery disease	1 (17)	2 (29)
Congestive heart failure	0	0
End stage renal failure	1 (17)	2 (29)
Malignancy in history	4 (67)	2 (29)
qSOFA criteria, n (%)		
SBP ≤ 100 mmHg	4 (67)	6 (85)
Respiratory rate ≥ 22 min^{-1}	1 (17)	6 (85)
GCS < 15	2 (33)	3 (60)
Suspected infection	2 (33)	7 (100)
Presence of surgery, n (%)	6 (100)	2 (29)
28-day mortality, n (%)	3 (50)	4 (57)
Thrombocyte count, (10^9 L^{-1})	168 [144 ; 285] *	261 [51 ; 395]

SBP, Systolic blood pressure; qSOFA, quick Sepsis Related Organ Failure Assessment; GCS, Glasgow Coma Scale**.** Values are given as median [IQR] or n (%)
***** Laboratory results were not available of one patient ([n=9])

In vivo mitochondrial assessment

The ALA-plaster was applied a median of 5.5 [5.0 ; 6.4] hours to the skin preceding the COMET monitor measurement. In 2 patients, quality of signal was not sufficient, so the ALA-plaster was replaced on the skin for 2 hours. The final attempt later that day was successful in only one of these patients, while both had an application time of over 7 hours. The first measurement was conducted 19.8 [15.5 ; 23.2] hours after admission to the ICU for both groups.

The median baseline mitoPO$_2$ for the first and second measurement in the control group were 61.8 [54.0 ; 70.4] and 64.5 [60.8 ± 73.6] mmHg, respectively. The mean baseline mitoPO$_2$ for the sepsis group was 57.6 [56.4 ; 73.4] mmHg and 69.9 [65.4 ; 74.4] for the first and second measurement respectively. (Figure 2A)

The mitoVO$_2$ for the first and second measurement in the control group were -6.4 [-4.5 ; -6.7] mmHg s^{-1} and -5.1 [-4.0 ; -6.0] mmHg s^{-1}. In the sepsis group an mitoVO$_2$ was measured of -7.3 [-3.5 ; -8.5] mmHg s^{-1} and -5.4 [-3.0 ; -7.9] mmHg s^{-1} for the first and second measurement respectively.

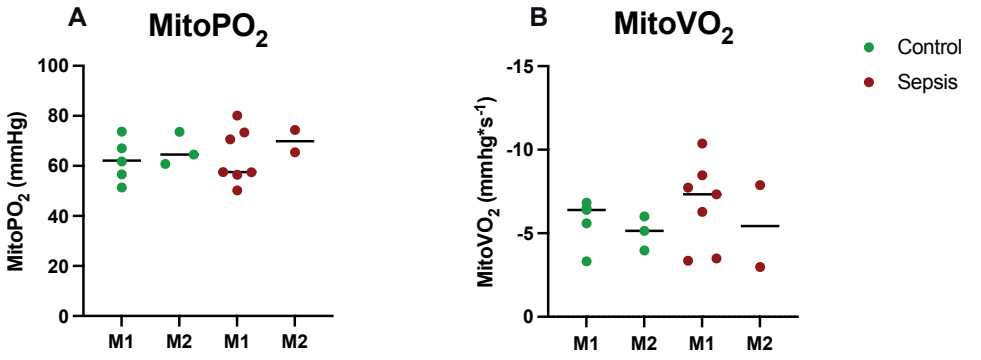

Figure 2 Results of COMET monitor measurements. Median and individual values. M1 is the measurement at ≤ 24 hours after admission to the ICU and M2 5 – 7 days after the first measurement **(A)** Median baseline mitochondrial oxygen tension (mitoPO$_2$) of the first and second measurement of the control group [n = 5] and [n = 3] and sepsis group [n = 7] and [n = 2] in **(B)** Median mitochondrial oxygen consumption rate (mitoVO$_2$) of the first and second measurement of the control group [n = 6] and [n = 5] and of the sepsis group [n = 7] and [n = 2] in mmHg.

Ex vivo platelet respiration

The respiration measurements on platelets were performed a median of 20.4 hours after ICU admission. The final platelet count in the O2k chamber was a median of 56 [23.5 : 110] * 10^6 platelets / mL. Following an average of 7 FCCP additions maximal respiratory rate was reached. We found an unstimulated oxygen consumption of 5.2 [1.4 ; 8.0] pmol/(s* 10^6 platelets) and 6.0 [4.2 ; 15.9] pmol/(s* 10^6 platelets) for the control and sepsis measurements respectively. An state 4o of 0.1 [-3.8 ; 2.0] pmol/(s* 10^6 platelets) and 2.9 [1.6 ; 14.0] pmol/(s* 10^6 platelets) and maximal flux of 13.7 [11.7 ; 16.4] pmol/(s* 10^6 platelets) and 28.7 [3.9 ; 53.0] pmol/(s* 10^6 platelets) for control and sepsis respectively as shown in figure 3A for measurement at admittance and figure 3B for measurement at 5-7 days after the first measurement.

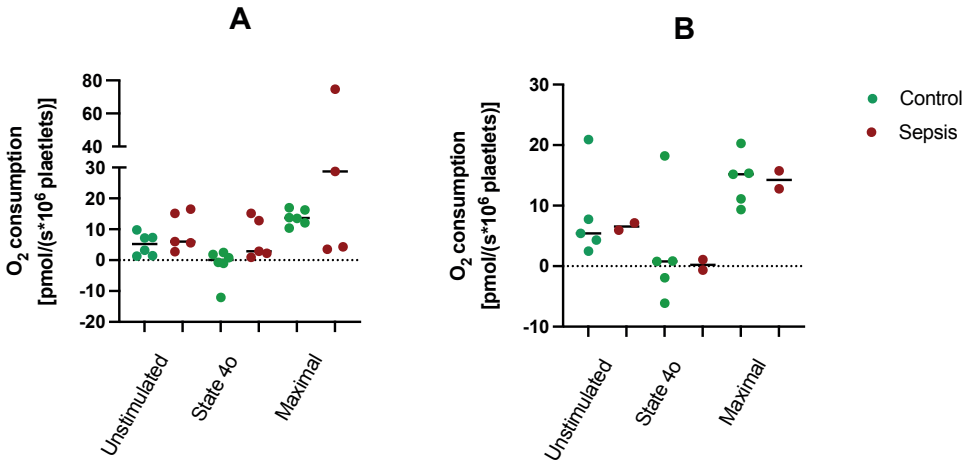

Figure 3 Thrombocyte respiration rates, median and individual values. **(A)** Measurement on day of admittance for the control [n = 6] and sepsis group [n = 5] **(B)** Measurements at 5 – 7 days after the first measurement of the control group [n = 5] and of the sepsis group [n = 2].

Figure 4 shows the mtDNA levels control groups on M1 (day of admittance) and M2 (5-7 days after first measurement). mtDNA levels are elevated above the threshold value of 3200 copies/ml in one control and 4 sepsis patients on the first measurement and for one control patient on the second measurement.

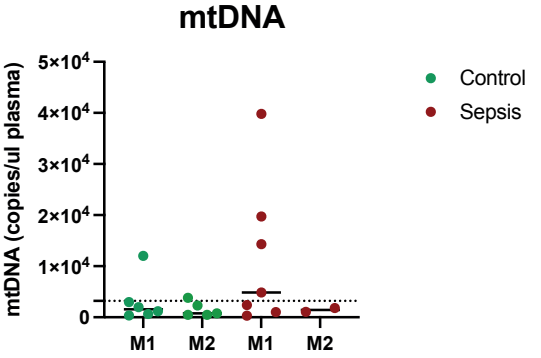

Figure 4 mtDNA measurements, median and individual values. mtDNA for the control group on day of admittance (M1, n = 6) and after 5-7 days (M2, n= 5) and for the sepsis group on day of admittance (M1, n = 7) and after 5-7 days (M2, n =2). The dotted line is at 3200 copies /microliter plasma.

DISCUSSION

We demonstrate the feasibility of the COMET monitor to measure mitochondrial oxygenation parameters in a very dynamic situation during the first day of admittance on the ICU ward. Of all the available patients, one out of 18 measurements failed with COMET, two out of 18 measurements failed in the *ex vivo* platelet respiration measurements. The average time of the first measurement after ICU admittance is close to 20 hours. Stabilization of patients with sepsis usually starts on the admitting ward and continues during the first hours of ICU admittance. We were interested in the onset of sepsis and its effect on mitoPO$_2$ and mitoVO$_2$ measurements, but we must conclude that the window of measurement in this dynamic period was probably missed.

The study was conducted over a period of more than two years, but we were able to include only 11 patients whom were eligible for data analysis. An in depth analysis of the failure to reach the initial interim analysis numbers follows. In short, a combination of unavailability of a dedicated researcher and a low frequency of sepsis patients on our ICU ward in a tertiary university hospital seemed to be the most pressing issues. The initial target of 10 patients in both groups, required to perform an interim analysis, was not achieved, and given the small sample size, further analysis as stipulated in the secondary research goals were not deemed feasible. We would like to further analyze the working of this study and the methods used to give advice for support when interested in studying the initiation phase of sepsis in ICU patients.

Lessons learned

Inclusion of sufficient numbers of eligible patients is a critical step in an observational study like this. In our ICU, researchers receive fulltime support for including candidates in their studies, however in a setting with multiple active protocols competing for the same subjects, and specific inclusion criteria such as the ones in this study limiting the number of eligible subjects, there is a real risk of not achieving sufficient eligible patient inflow. One way to mitigate this challenge is to include a dedicated researcher in the screening flow for the study of candidates.

Another challenge relating to recruiting sufficient subjects in this study is related to the organization of ICU care within the hospital and the region. Our ICU is a third level ICU, which implies that it serves, asides from being a primary ICU, as a second-opinion hospital for neighboring ICU's. As a consequence, a number of patients coming into the ICU with severe health challenges, such as difficult ventilation patients, were not eligible

for enrollment in our study, given that they had already passed the initiation phase of sepsis. Additionally, third-level ICU's serve a relatively complex patient population. Hematologic oncology patients for example, whom suffer from bone marrow depletion during their treatments. A relatively simple infection in this group could have disastrous consequences These subjects fulfill the criteria of sepsis, however oncology-targeted therapies are likely to affect mitochondrial function as well and as a consequence these patients cannot be included in the study. Furthermore, during oncology treatment, bone marrow depletion is a common side effect and the accompanying thrombopenia limits the ex vivo platelet respiration experiments. Lastly, the challenges around the limited screening window resulted in a number of eligible subjects not included in our study. Intensive care is by its definition a 24/7 facility, however limited availability of the research nurse or the dedicated researcher, both of whom are critical for performing the informed consent procedure and preparing the COMET measurements, meant that not all eligible patients were admitted into this study.

Evaluation of methods

The informed consent method was well thought of in advance with expert help of the research unit of the ICU and the expert opinion of our ethicist Dr. Kompanje. However the 4 possible ways of receiving informed consent in a study with a relative low frequency of inclusion were not efficient. The follow up of patients after ICU admittance for final informed consent of the primary subject, if the patient was outplaced to another hospital follow up was difficult and not enough attention for follow up and receiving informed consent lead to exclusion of 5 patients.

The design of this study was centered around the *in vivo* measurement of mitochondrial function in the initiation phase of sepsis. But due to the organization of the study protocol study participants were only available after admittance to the ICU and since these patients were in a critical phase of ICU care direct application of the ALA plasters was not always possible during this essential phase of treatment. Furthermore 5 – 7 hours of ALA application necessary to induce sufficient levels of PpIX further lengthened the time before the first measurement. A possible solution would be to make the ALA plasters part of the admittance protocol for the ICU or even better finding a different plaster technique which shortens the application time of the plaster significantly.

The O2C measurement of microvascular flow and oxygenation has been used due to the ease of application and short onset time of this method seemed perfect as screening for microvascular anomalies. In our study protocol lengthy assessment of the patient was

not necessary leading to a median measurement time of the O2C monitor of shorter than 30 minutes. Since this methods use is aimed at measuring changes in a patient over time we think the short measurement time does not lead to reliable data. Using the O2C for the rest of the protocol will not give any advantage in the current setup and therefore our advice is to abandon the O2C measurements when only measuring for short periods of time.

Measuring changes of mitochondrial function in platelets is possible, however thrombopenia limited the use of this technique. When interested in the pathophysiological changes of mitochondrial function in the different phases of sepsis, measurement of these changes *in vivo* and *ex vivo* will give a more complete picture of the changes affecting the patient. In a more longitudinal assessment of these pathophysiological changes a reliable measurement of mitochondrial change to compare to the changes measured with the new monitor is essential. A problem with the *ex vivo* measurements is the targeted time, with a maximum of 1 hour, between measurement and blood sampling, and therefore this method is only possible when the measurements take place in the same hospital.

mtDNA measurements out of serum plasma is technically advanced but can be performed centrally and grouped in a dedicated session and therefore might be the easiest way to measure mitochondrial damage when performing a multi-centre study, this might be of an advantage when the *in vivo* measurements are linked to mitochondrial damage and as a marker for mitochondrial damage mtDNA levels are used.

In short, our advice is when a researcher is interested in the pathophysiological changes of mitochondrial function in sepsis a more longitudinal approach will probably be advantageous, possible with multiple measurements over the first days. Using the advanced monitoring the ICU ward in our tertiary university hospital provides in combination with an assessment tool of microvascular flow. *Ex vivo* assessment of mitochondrial function in blood cells (white blood cells or platelets) would be of additional value in such an protocol.

When interested in the distribution of the *in vivo* parameters of mitoPO_2 and mitoVO_2 during sepsis or an ICU admittance a multi-centre approach or a primary centre other than the Erasmus Medical Centre provides another casemix, a higher patient turnover and therefor a shorter protocol time. mtDNA measurements in this protocol would be efficient and easy to implement and is strongly linked to survival in earlier studies. *Ex vivo* mitochondrial function assessment is limited in this design due to the limited time available to asses these changes in the Oroboros. In this protocol short use of the O2C gives insufficient additional value in our view.

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Chapter 8

Monitoring of mitochondrial oxygen tension in the operating theatre: an observational study with the novel COMET® monitor

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Abstract

Introduction The newly introduced Cellular Oxygen METabolism (COMET®) monitor provides a means to measure mitochondrial oxygenation (mitoPO$_2$) using the protoporphyrin IX triple state lifetime technique (PpIX-TSLT). In this study we aim to investigate the feasibility and applicability of the COMET® measurements in the operating theatre during stable operating conditions. Furthermore, two cases are presented wherein the characteristics of the mitoPO$_2$ measurements are shown in unstable conditions.

Methods: In this observational study mitochondrial oxygenation was measured in 20 patients during neurosurgery using the COMET® measuring device. Tissue oxygenation and local blood flow were measured by the Oxygen to See (O2C). Primary outcomes included mitochondrial oxygen tension, skin temperature, mean arterial blood pressure, local blood flow and tissue oxygenation.

Results: Mean application time of the ALA plaster was 17 ± 3.3 hours. Mean baseline mitoPO$_2$ was 60 ± 17 mmHg, mean mitoPO$_2$ remained between 40-60 mmHg during surgery.

Conclusion: This study has presented mitochondrial oxygenation measurements during both stable and unstable hemodynamic conditions in the operating theatre. The results demonstrate the reliability of mitochondrial oxygenation as measured by the COMET® monitor and provide the first step for future research into their added value during major surgery.

Introduction

Safeguarding an **adequate tissue oxygenation to** ensure aerobic metabolism **is an** important task of an anesthesiologist during general anesthesia. After inhalation of air, oxygen enters the lungs, where it passes through the alveoli into the circulating blood where it binds to hemoglobin. Oxygen is then delivered to tissue cells via macro- and microcirculatory flow and finally by the diffusion of molecular oxygen. Eventually the oxygen molecule reaches its ultimate destination, the mitochondria. Here oxygen is used in the oxidative phosphorylation to produce adenosine triphosphate (ATP) that acts as the main energy source for many cellular processes. Current clinical management of maintaining adequate tissue oxygenation is mainly focusing on the normalization of systemic hemodynamic parameters such as blood pressure, hemoglobin levels, peripheral oxygen saturation, cardiac output and venous saturation. But insight into oxygenation where it matters the most, inside the mitochondria, was not available for clinical use until recently.

So far, our knowledge about mitochondrial physiology and oxygenation is obtained from ex vivo measurements on isolated mitochondria and cells, mainly derived from muscle biopsies. Repeated sampling and continued monitoring of mitochondrial parameters in humans is not possible because of ethical and technical restraints [1]. The protoporphyrin IX triple state lifetime technique (PPIX-TSLT), based on delayed fluorescence of protoporphyrin IX can overcome these limitations [2,3]. It enables non-invasive mitochondrial oxygen tension ($mitoPO_2$) measurements in humans [4-6]. The positive results of a feasibility study in healthy human volunteers [7,8] resulted in the development of the clinical measuring device "COMET®", an acronym for Cellular Oxygen METabolism [5]. The introduction of COMET® paved the way for the next step in the development of a monitoring technique, which measures mitochondrial oxygenation during surgery.

In this longitudinal observational study we studied the usability and performance of the COMET® under real-life circumstances during surgery. We focused especially on the behavior of the new parameter $mitoPO_2$ during stable hemodynamic circumstances, and hypothesized that $mitoPO_2$ would be stable over time under such conditions. To investigate the stability of the $mitoPO_2$ measurements we chose to perform the measurements in patients undergoing neurosurgical procedures. We made this choice because of the relatively healthy patient population, stable operation conditions and the relatively long operation duration. As contrast to the behavior of $mitoPO_2$ under the study circumstances, we present a number of cases from another ongoing study, in which we

show the dynamics of mitoPO$_2$ under unstable hemodynamic conditions during acute blood loss and hypovolemia.

Methods

Trial design, setting, participants

The study was approved by the Institutional Review Board (IRB) at the Erasmus Medical Center CCMO-register 'Non-invasive monitoring of mitochondrial oxygen consumption and oxygenation (COMET): observational clinical study', (NL51937.078.15). The additional cases also come from an IRB-approved study running in the Erasmus Medical Center ' Noninvasive measurement of mitochondrial oxygen tension (mitoPO$_2$) compared to continuous hemoglobin-monitoring during major surgery.' (NL63158.078.17). All study procedures were performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained from all patients prior to study participation.

This single center observational study was performed at the Erasmus Medical Center, a tertiary care center in the Netherlands. Inclusion ran from April 2016 up until March 2020. Patients aged between 18-70 years old and scheduled for neurosurgery were considered eligible for participation. Exclusion criteria included surgeries without need for invasive intra-arterial blood pressure monitoring, presence of mitochondrial diseases and pregnancy or lactation. The goal of the study was to measure mitoPO$_2$ in the operation room under stable conditions. Therefore, patients were excluded if hemodynamic unstable situations during surgery occurred: 1) if blood transfusion was required, 2) if the mean arterial pressure (MAP) decreased more than 25% from baseline or 3) if high doses of vasopressors were needed (noradrenaline ≥0,10 mcg/kg/min or the equivalent dose of phenylephrine.) The raise in blood pressure during the hemostasis, induced by increasing the rate of infusion noradrenaline or phenylephrine is according to standard procedure and is not taken into account for the analysis of the results

Patient screening was performed by the research team, based on data collected at the standard care perioperative consult. Written informed consent was obtained from all patients prior to study participation.

Principle of mitoPO2 measurements

The background and principles of the PpIX-TSLT are described in detail elsewhere [2,5,9]. In short, PpIX is the final precursor of heme in the heme biosynthetic pathway. PpIX

is synthesized in the mitochondria, and administration of 5-aminolevulinic acid (ALA) substantially enhances the PpIX concentration. PpIX possesses a triplet state that reacts strongly with oxygen, making its lifetime oxygen-dependent. Population of the first excited triplet state occurs upon photo-excitation with a pulse of light, and causes the emission of red delayed fluorescence. The delayed fluorescence lifetime is related to mitoPO_2 according to the Stern-Volmer equation:

$$PO_2 = \frac{\frac{1}{\tau} - \frac{1}{\tau_0}}{k_q}$$

in which τ is the measured delayed fluorescence lifetime, k_q is the quenching constant and τ_0 is the lifetime at zero oxygen. The Stern- Volmer equation is valid for a homogenous oxygen distribution and after excitation with a pulse of light of which the lifetime is much shorter than τ. In case of a nonhomogenous oxygen distribution inside the measurement volume, a reliable estimation of the average PO_2 can be made by the rectangular distribution method (RDM) [10,11].

The signal/noise ratio (SNR) of resulting traces was calculated and defined as the ratio of maximum signal amplitude to the peak-to-peak noise. COMET® evaluates signal quality, which is calculated from the SNR value; an increase of one in SNR is approximately 1% in signal quality up till a SNR of 50. Lifetime analysis operates stably at moderate signal quality (>20%) [5]. Therefore, only delayed fluorescence signals with a signal quality > 20% were analysed and included in the present dataset.

Procedures (Measurement)

Induction of anesthesia was performed based on the attending anesthesiologist's preference. Intraoperative monitoring, ECG based heart rate, invasive blood pressure, body temperature, skin temperature and oxygen saturation, inspired oxygen fraction (FiO_2), anesthesia infusion rate and vasopressor pump setting were part of the standard monitoring and were stored in the electronic patient data management system. All patients were kept normothermic with use of a warm air blanket. Normovolemia was pursued with intravenous crystalloids based on the pulse pressure variation index (threshold above 13).

Oxygen measurements were performed by means of the COMET® monitor (Photonics Healthcare, Utrecht, the Netherlands). A self-adhesive patch containing 8 mg ALA (Alacare, photonamic GmbH und Co. KG, Pinneberg, Germany) was applied on the sternal skin, for induction of PpIX. The Alacare patch is still under investigation for FDA approval for the intended use of the COMET® measurement. To enhance ALA penetration adequate skin preparation proved essential. Hair was shaved (if present) and the skin was rubbed with a fine abrasive pad of a standard ECG sticker to remove the top parts of the stratum corneum. During ALA application, the skin was protected from light by the patch for minimal 5 hours. During these 5 hours a suitable concentration of PpIX was synthesized to enable measurements of mitoPO$_2$. After induction of anesthesia, the ALA patch was removed and the measuring probe applied to the ALA-treated skin. The mitoPO$_2$ was automatically measured every 5 minutes during the operation.

In addition to mitoPO$_2$, tissue oxygenation saturation and perfusion parameters were measured intraoperatively (O2C, oxygen to see version 2424, Lea Medizintechnik GmbH, Giessen, Germany). The O2C measures three parameters: The local capillary venous saturation (StO$_2$), the local velocity of blood given in velocity units (VU) and the local micro vascular blood flow given in flow units (AU). Both the COMET® Skin Sensor and the O2C probe (LFX-43) were positioned on the sternum next to each other.

All measurements were performed from the start of surgery until the end of surgery, in order to exclude the effects of induction of anesthesia and the accompanying influences of medication and preoxygenation on mitochondrial and microvascular parameters. To observe the applicability and stability of the mitoPO$_2$ measurements the primary outcome measures included mitoPO$_2$ (mmHg), flow (AU), probe temperature (degrees Celsius) and MAP (mmHg) over time.

Statistical analysis

A sample size calculation was performed using G*Power software and was based on previous research in healthy volunteers [7,12]. Because of technical improvements less device-induced variation was expected and therefore a lower standard deviation was assumed. MitoPO$_2$ was suspected to be slightly higher due to the continuous oxygen supplementation during surgery. A sample size of 20 patients was calculated with an assumed mean difference of 12 mmHg and standard deviation of 18 mmHg, a type I error probability of 0.05 and a power of 80%.

Statistical analyses were performed using Graphpad Prism 8 and IBM Statistics SPSS 26. Mitochondrial oxygen tension measurements with a signal quality under 20% were not included. As the O2C measures several times a minute the data was averaged over 60 seconds to allow fluctuation in the minutes range. Descriptive statistics were used to describe demographic parameters. Continuous variables are described as mean and standard deviation (SD)).

Results

A total of 24 neurosurgery patients were included in the study, 4 patients were withdrawn. One patient was excluded from further analysis because of insufficient signal quality, one patient had forgotten to apply the ALA patch before surgery, in one patient the operation was rescheduled due to the intervention of an emergency operation, in one case the positioning during surgery changed from back to prone position after inclusion. In 2 of the remaining 20 patients the O2C data is missing because of the unavailability of the monitor. Surgical and patient characteristics are summarized in table 1. On average, the ALA patch was applied 17 ± 3.3 hours before surgery. After the measuring surface was well shielded from light, sufficient signal quality was measured at the beginning of surgery. Incidentally, signal quality was below 20% which led to exclusion of one or more data points in 6 out of 20 patient and a total of 3.5% of the mitoPO$_2$ data points.

Table 1. Baseline patient characteristics and clinical data

Variables	Total (n=20)
Age (yr)[a]	56.2 (± 11.7)
Sex	
Male/female	10/10
BMI[a]	26.8 (± 3.7)
Medical history	
Chronic obstructive pulmonary disease	10% (2)
Hypertension	15% (3)
Diabetes Mellitus	5% (1)
Vascular event	22% (4)
Surgery type	
Resection of a vestibular schwannoma	2/20
Hypophysectomy	6/20
Trepanation	12/20
Dexamethason use before surgery	40% (8)
ASA I/II/III/IV	4/8/7/1
Blood pressure[a] (mmHg)	
Systolic	134.4 (±15.2)
Diastolic	80.7 (±10.5)
Heartrate (beats/min)[a]	74.8 (± 15.4)
SpO_2 (%)[a]	97.4 (± 1.43)
Blood loss (ml)[a]	217 (± 202)

[a] = mean (standard deviation)
Abbreviations: ASA, American Society of Anesthesiologists; BMI, body mass index; SpO_2, oxygen saturation

All 20 patients were hemodynamically stable during surgery, as described in the inclusion criteria. In all patients low doses vasopressors were needed to maintain MAP above 65 mmHg. In 14 patients noradrenaline was administered (0.05 mcg/kg/min ± 0.02 mcg/kg/min) and phenylephrine was used in 6 patients (0.28 mcg/kg/min ± 0.14 mcg/kg/min). No rises in serum lactate level were observed. MAP, microcirculatory blood flow and tissue oxygen saturation were stable during surgery as presented in figure 1 (A, C and E). The mean skin temperature changed from baseline (start surgery) over time (figure 1B). In the first hour of operation, the mean skin temperature increased with more than 1°C caused by a number of patients whose skin temperature rose more than two degrees. Baseline mitoPO$_2$ (± SD) was 60 ± 17 mmHg, and during surgery the mean mitoPO$_2$ remained between 60 and 40 mmHg but a slow decline from baseline was observed during the first hour of surgery (figure 1D).

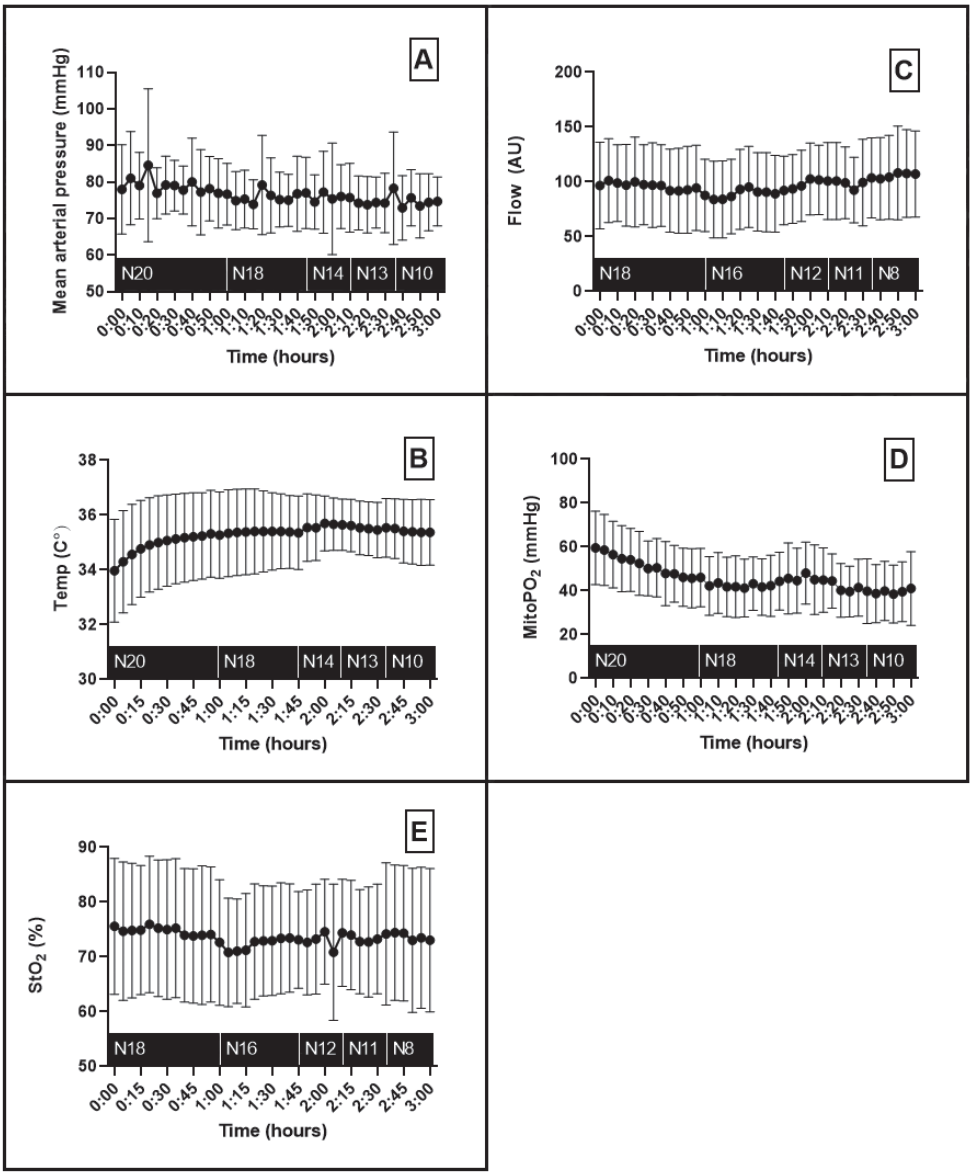

Figure 1: Overview of the hemodynamics parameters of the included patients during neurosurgery. A. Mean Arterial Pressure B. Mean skin temperature. C. Mean flow in microcirculation measured with the O2C. D. Mitochondrial oxygen tension as measured by the COMET. E. Tissue oxygen saturation as measured by the O2C. Data presented as mean (dot) and ± standard deviation.

MitoPO2; mitochondrial oxygen tension, StO2; tissue oxygen saturation, AU; arbitrary units, MAP; mean arterial pressure, Flow; microcirculatory blood flow, Temp; skin temperature

In figure 2 a single case of the neurosurgery cohort is presented. This case is notable because of long operation time. A 35-year-old woman who had surgery on a difficult-to-reach vestibular schwannoma had surgery for more than six hours. She remained hemodynamically stable throughout the operation, no vasopressors were administered during surgery. A total of 600ml blood was lost, which was supplemented with crystalloid and colloid solutions to maintain a normovolemic fluid balance. Figure 2 shows the possibility to measure stable mitoPO$_2$ during long period of time under stable hemodynamic conditions without losing signal quality.

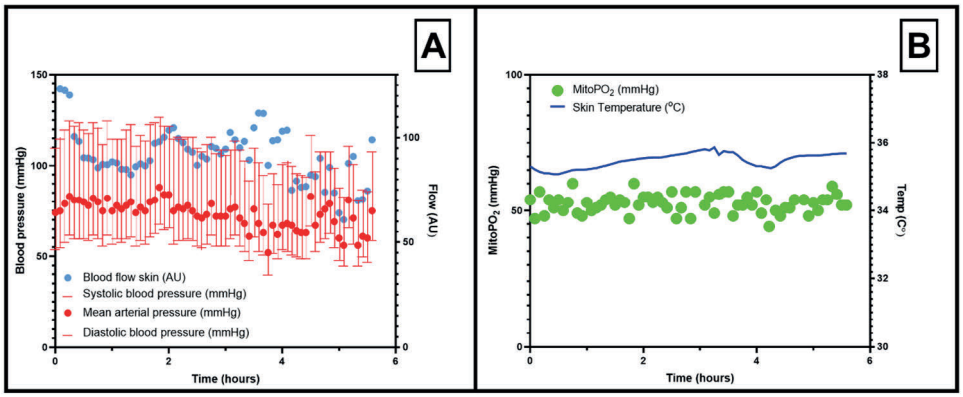

Figure 2: Representative case of stable mitoPO2 measurements during an operation of more than six hours.

MitoPO2; mitochondrial oxygen tension, AU; arbitrary units, Temp; skin temperature

Cases

Case A: pancreaticoduodenectomy with significant blood loss

A 61-year-old ASA II male underwent an open pancreaticoduodenectomy according to WHIPPLE procedure because of a suspected cholangiocarcinoma. Except a previous cholecystitis for which several ERCP procedures and a laparoscopic cholecystectomy has been performed, no other details in his medical history were revealed. No medication

was used before surgery. Preoperative physical, abdominal, respiratory and cardiac examination were unremarkable. The blood testing results are summarized in table 2.

Table 2. Preoperative laboratory assessment

Laboratory test (unit)	Case A	Case B	Ref. value
Glucose (mg/dL)	185.4	102.6	72.0-109.8
Creatinine (mg/dL)	0.78	0.83	0.74-1.30
Hemoglobin (g/dL)	16.3	14.2	13.9-16.9
Platelets (cells/mm^3)	291.000	245.000	150.000-370.000
Leukocytes (cells/mm^3)	6.6000	3.5000	3.5000-10.000

An epidural catheter was inserted before induction of anesthesia and epidural analgesia with ropivacaine/sufentanil was given prior to surgery. For induction of anesthesia, an IV bolus of 250 mg propofol, 50 mg rocuronium and infusion of remifentanil 9 mcg/kg/h was used. Anesthesia was maintained by continuous infusion of propofol 8 mg/kg/h and remifentanil 8 mcg/kg/h. Directly after induction, continuous infusion of noradrenalin (0.05-0.19 mcg/kg/min) was necessary to maintain an adequate blood pressure. On top of standard perioperative monitoring consisting of invasive blood pressure measurements, peripheral oxygen saturation, electrocardiography, and temperature measurements, COMET® was used for mitoPO_2 measurements, and continuous total hemoglobin (SpHb) measurements were performed with a disposable Masimo Rainbow adult adhesive sensor (R2-25a Masimo Corporation, Irvine, USA). Because of a tumor location near the portal vein the procedure was complicated and in a period of 8 hours the patient lost 3750 ml of blood (figure 3). Resuscitation of blood loss was performed using crystalloid (NaCl 0,9% and Sterofundin), colloid solution (Voluven 6%) and vasoactive medication (figure 3). Eventually, intraoperatively there seemed no need for a blood transfusion and intraoperative serum lactate remained low. At the end of surgery fluid balance was 1390 ml positive. During surgery, the mitoPO_2 value gradually declined from 79 mmHg to 5 mmHg. (figure 3). This decrease in mitoPO_2 was accompanied by a decline in hemoglobin from 14.82 g/dL to 9.9 g/dL (determined by a blood gas analyzer). Continuous SpHb measurements were also executed and declined from 11.76 g/dL to 10.63 g/dL. Post-operatively the patient developed increased lactate levels and eventually received blood transfusion.

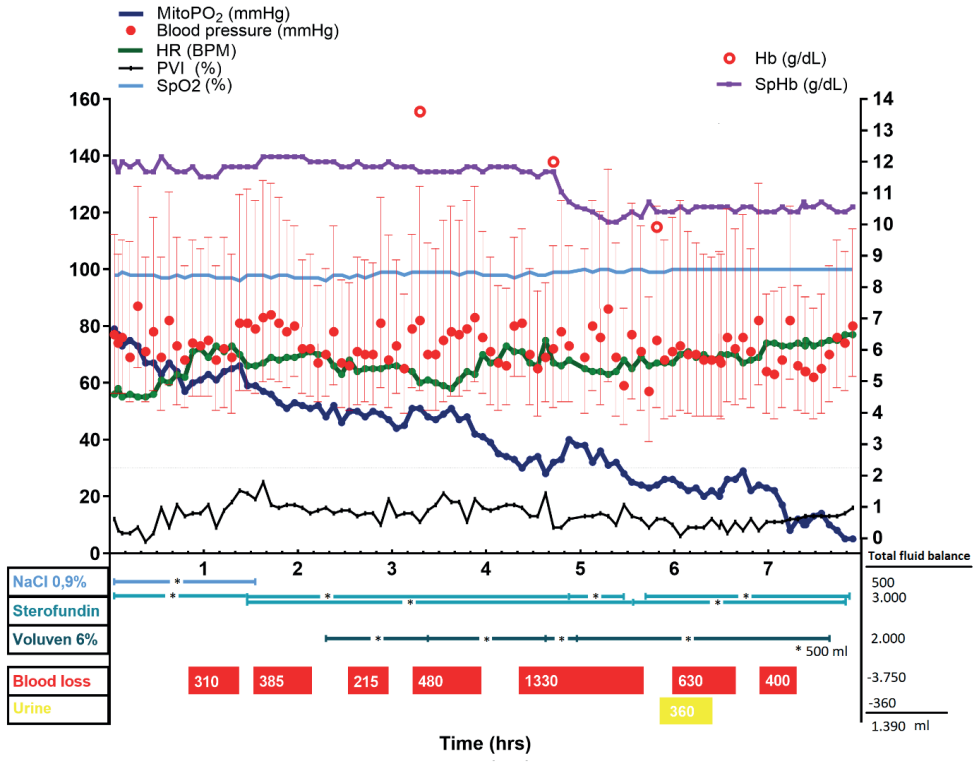

Figure 3: Representative case with significant amount of blood loss during pancreaticoduodenectomy. Blood pressure is presented in mean arterial pressure (dot) with the diastolic and systolic blood pressure represented by the bottom and top whiskers.
MitoPO2; mitochondrial oxygen tension, Hb; hemoglobine, SpHb; peripheral hemoglobin, SpO2; peripheral oxygen saturation, PVI; pulse variation index, HR; heart rate

Case B: Partial hepatectomy

A 57-year-old women, ASA I underwent a partial hepatectomy because of a rapidly growing cyst. The patient's relevant history includes a possible transient ischemic attack (TIA) in 2017. Her medication included clopidogrel 75mg once a day, which was stopped 5 days prior to surgery. Preoperative abdominal, respiratory and cardiac examination were unremarkable. The blood testing results are summarized in table 2.

An epidural catheter was inserted before induction of anesthesia and epidural analgesia with ropivacaine/sufentanil was given prior to surgery. For induction of anesthesia, an IV bolus of 140 mg propofol, 50 mg rocuronium and infusion of remifentanil 7 mcg/kg/h was used. Anesthesia was maintained by using sevoflurane and continuous infusion of

remifentanil 5 mcg/kg/h and epidural analgesia. After induction, continuous infusion of noradrenalin, starting at 0.30 mcg/kg/min and gradually increasing to 0.60 mcg/kg/min, was necessary to maintain an adequate blood pressure. On top of standard perioperative monitoring, consisting of invasive blood pressure measurements, peripheral oxygen saturation, electrocardiography, temperature measurements and central venous pressure, COMET® was used for mitoPO$_2$ measurements, and continuous total hemoglobin (SpHb) measurements were performed with a disposable Masimo Rainbow adult adhesive sensor.

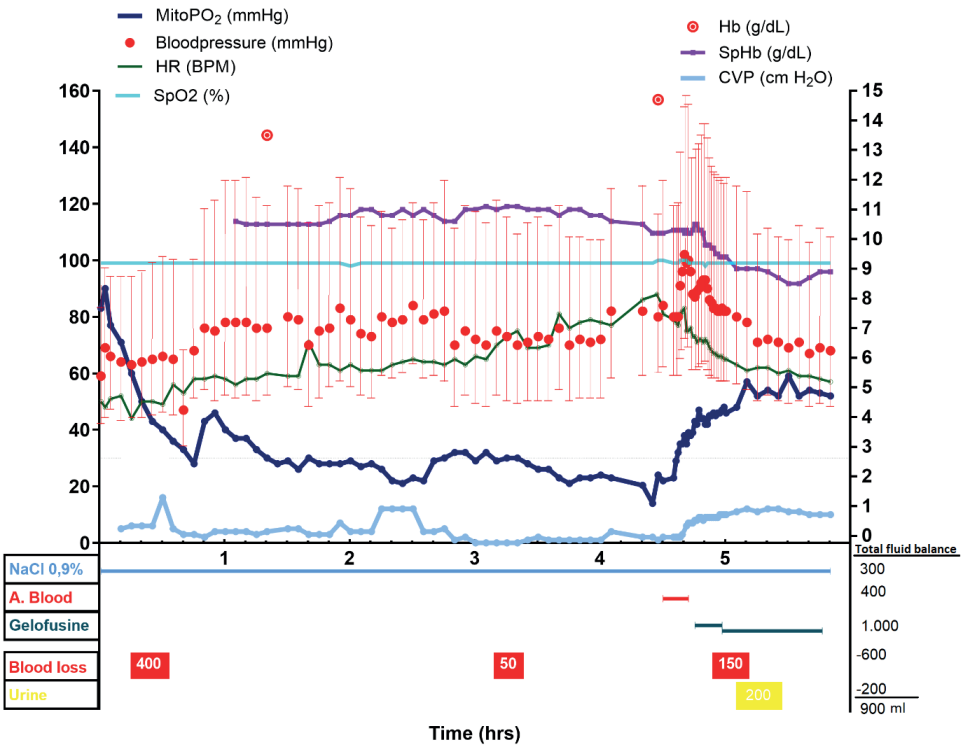

Figure 4: Representative case of a patient with controlled hypovolemia and recovery to normovolemia.

At the beginning of the surgery, 400 ml of blood was withdrawn to preserve a low central venous pressure. For the same purpose, fluid admission was kept to a minimum throughout the surgery. Only when the hepatectomy was done, fluids were given starting with an autologous blood transfusion followed by two times 500 ml of Gelofusine (figure

4). Fluid balance was 900 ml positive. Apart from 400ml withdrawal for autologous transfusion, 200ml blood was lost during surgery. Under this controlled hypovolemic circumstance, mitoPO$_2$ dropped from 83 mmHg to values around 20 mmHg. Lactate levels rose from 9.0 mg/dL to 19.8 mg/dL. After the tumor was removed and volume status was restored to a normovolemic state, mitoPO$_2$ showed recovery.

Discussion

The results of this study show that it is feasible to measure mitoPO$_2$ with the COMET® during surgery in a real-world situation. It is important to note that covering the measurement surface and protecting it against ambient light is essential for getting a good signal quality.

In hemodynamically stable patients a mean mitoPO$_2$ between 60-40 mmHg was found and baseline mitoPO$_2$ was 60 ± 17 mmHg. Previously published results in healthy volunteers showed mean mitoPO$_2$ (± SD) values of 44 ± 17 mmHg. Higher values were expected in this study, as a higher inspiratory oxygen fraction was used during surgery. Since mitochondrial oxygen tension reflects the oxygen balance between oxygen supply and oxygen demand [13,14], a decreased metabolism during general anaesthesia may also cause increased mitochondrial oxygen levels [15]. Not only during general anesthesia, but also with various lifestyle-related diseases like neurodegenerative disease [16], cardio-vascular disease [17] and obesity [18,19] a reduced oxygen metabolism and mitochondrial dysfunction are described. Septic patients are another example in whom a reduced oxygen metabolism and mitochondrial failure have been described [20]. Recently a study in patients with severe sepsis showed a relatively high mitoPO$_2$ (61 ± 10 mmHg) [21].

Despite the intention to measure mitoPO$_2$ in the operation theatre under stable conditions this was not achieved for all parameters in all patients. In some patients, skin temperature increased after the warm air blanket was applied. We hypothesize that this played a role in the small, probably not clinically relevant, drop in mitoPO$_2$ (figure 2). Theoretically, this drop can be explained by an increase in cellular metabolism due to a higher temperature resulting in a higher oxygen demand and lower mitoPO$_2$ values. As a result of this higher rate in energy consumption cells rapidly break ATP down to adenosine diphosphate (ADP) an inorganic phosphate group (Pi). In this process adenosine monophosphate (AMP) and [H^+] are released which are both strong vasodilators and blood flow and tissue oxygen saturation increases [22,23]. Our data is supporting this theory, as a rise in temperature gives a drop in intracellular PO$_2$ (mitoPO$_2$) while cutaneous microvascular blood flow and

capillary-venous oxygen saturation did not decline. A similar phenomenon of decreasing mitoPO$_2$ levels while capillary-venous oxygen saturation remained unchanged has been described earlier by Ubbink et al. [5] They observed a drop in mitoPO$_2$ after a decline in microvascular blood flow by a bolus of clonidine, but no changes in capillary-venous oxygen saturation were measured. An alternative explanation divergence between mitoPO2 and microcirculation could be the different wavelengths used by the three different measurement techniques. StO$_2$ is measured with the spectra absorbance of visible light, mitoPO$_2$ is measured with green light (515 nm) and velocity and flow are measured with the hemoglobin laser Doppler frequency shift red light. All these different wavelengths give different tissue penetrations [24] and thus measure in different tissue compartments and could therefore show different results. Anyhow, based on these findings it is clear that measuring oxygen availability directly at cellular level provides complementary data and new insight.

In the two cases we described the oxygen transport to the cells is at risk. In the first case major blood loss was followed by normovolemic hemodilution and a drop in haemoglobin levels occurred. This normovolemic hemodilution was accompanied by a slow decrease of the mitoPO$_2$ to values below 10 mmHg. Striking is that especially mitoPO$_2$ decreased while other usual parameters, such as blood pressure, heart rate and pulse variation index did not change. Hemoglobin levels and SpHb were reduced but not to values indicating a direct need for blood transfusion, and even serum lactate levels remained low in the intraoperative phase. This advocates the decision not to use blood transfusion despite the large amount of blood loss. However, in the postoperative phase lactate levels rose and the patient did need a blood transfusion. We have previously observed more or less the same behavior of mitoPO$_2$ in a single case during major abdominal surgery [25]. These cases suggest that mitoPO$_2$ monitoring can be of great value for improving hemodynamic management. In the second case presented in this study, a perioperative phase with controlled hypovolemic circumstances has been presented. The hypovolemic circumstance resulted in low central venous pressure (CVP) values and low mitoPO$_2$ values between 30 and 20 mmHg, whereas most other parameters remained stable. Only lactate levels had risen, indicating tissue hypoxia. These examples show that mitoPO$_2$ can clearly respond to hemodynamically unstable situations. Furthermore, it shows that during hemodynamically unstable conditions mitoPO$_2$ values that are really below the normal standard deviation of less than 10 mmHg have been measured. In the second case, in which hypovolemic conditions have been pursued for a longer period of time, also systemic signals of hypoxia were intraoperatively observed. In the first case,

a rise in serum lactate only became evident in the postoperative phase. These findings suggests that mitoPO$_2$ around and below 20 mmHg during a longer period of time can be associated with cellular hypoxia and systemic signs of anaerobic metabolism. The relationship between increased serum lactate and low mitoPO$_2$ has also been observed by Römers et al [26]. Since increased serum lactate is associated with adverse outcome, we hypothesize that additional mitoPO2 can be of added value during surgery [27].

Because many factors are involved in maintaining an adequate cellular oxygenation, it seem wise to not base perioperative hemodynamic, blood transfusion and fluid therapy strategies during complex surgery on standard intraoperative parameters alone. Therefore, we suggest to use additional mitochondrial oxygenation and microvascular flow measurements in major surgery and in blood transfusion management to prevent cellular hypoxia and organ damage to improve long-term outcome [6,7,28]. The added value of the mitochondrial oxygenation measurements during major surgery must be demonstrated further in future studies. The present study is a first step towards evidence based monitoring of mitochondrial oxygenation in the operation theatre.

This study shows the feasibility and applicability of measuring mitoPO$_2$ in the operating theatre using the COMET® monitor. MitoPO$_2$ measurement remains feasible even during long-lasting surgery.

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Declarations

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Dr. Mik is founder and shareholder of Photonics Healthcare B.V., Utrecht, The Netherlands. Photonics Healthcare B.V. holds the exclusive licenses to several patents related to mitochondrial oxygen measurements, filed and owned by the Academic Medical Center in Amsterdam and the Erasmus Medical Center in Rotterdam, The Netherlands. Photonics Healthcare B.V. is the developer and manufacturer of the COMET® device. The remaining authors declare no competing interests.

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Authors contributions

FH: study design, inclusion of patients, measurements, data processing, writing article.

LS: inclusion of patients, measurements, data processing, writing article.

MWB: inclusion of patients, measurements, data processing.

LR: study design, inclusion of patients, measurements, data processing.

RJ: study design, inclusion of patients.

RJS: Head of the department, editing of manuscript.

EM: study design, head of research team, editing of manuscript.

All authors read and approved the final manuscript.

Chapter 9

Mitochondrial oxygen monitoring during surgical repair of congenital diaphragmatic hernia or esophageal atresia: a feasibility study

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Abstract

Current monitoring techniques in neonates lack sensitivity for hypoxia at cellular level. The recent introduction of the non-invasive Cellular Oxygen METabolism (COMET) monitor enables measuring in vivo mitochondrial oxygen tension(mitoPO$_2$), based on oxygen-dependent quenching of delayed fluorescence of 5-aminolevulinic acid (ALA)-enhanced protoporphyrin IX.

The aim is to determine the feasibility and safety of non-invasive mitoPO$_2$ monitoring in surgical newborns. MitoPO$_2$ measurements were conducted in a tertiary pediatric center during surgical repair of congenital diaphragmatic hernia or esophageal atresia. Intraoperative mitoPO$_2$ monitoring was performed with a COMET monitor in 11 congenital diaphragmatic hernia and 4 esophageal atresia neonates with the median age at surgery being 2 days(IQR 1.25-5.75). Measurements were done at the skin and oxygen-dependent delayed fluorescence was measurable after at least 4 hours application of an ALA plaster. Pathophysiological disturbances led to perturbations in mitoPO$_2$ and were not observed with standard monitoring modalities. The technique did not cause damage to the skin, and seemed safe in this respect in all patients, and in twelve cases intraoperative monitoring was successfully completed. Some external and potentially preventable factors – the measurement site being exposed to the disinfectant chlorohexidine, purple skin marker or infrared light - seemed responsible for the inability to detect an adequate delayed fluorescence signal. In conclusion, this is the first study showing it is possible to measure mitoPO$_2$ in neonates and that the cutaneous administration of ALA to neonates in the described situation can be safely applied. Preliminary data suggests that mitoPO$_2$ in neonates responds to perturbations in physiological status.

Keywords: mitochondria$_1$, oxygen$_2$, neonate$_3$, surgery$_4$, monitoring$_5$

Introduction

Major (non-cardiac) neonatal surgery is challenging for clinicians. The neonatal homeostasis is a frail equilibrium and is highly affected by general anesthesia and surgical manipulation (1,2). The anesthesiologist aims to monitor the physiology with the help of the heart rate, invasive blood pressure, saturation, end-tidal carbon dioxide, skin perfusion, urine output and serum lactate. These broad range of monitoring modalities are used as surrogate of end-organ perfusion with adequate oxygen transport as a prime goal. To date, the optimal blood pressure in neonates for adequate perfusion of peripheral and cerebral tissue is unknown. Invasive techniques available for effective monitoring of the circulation/cardiovascular system are seldom used due to technical restraints in neonates or are simply not feasible during neonatal surgery (3). Yet, the incidence of brain injury after (non-cardiac) neonatal surgery is increasingly reported (4)(5) as well as altered long–term neurodevelopmental outcomes (6–9). Several factors are thought to contribute to the postoperative brain injury, including alterations in the perioperative neonatal hemodynamics.

Adequate oxygen supply to tissues is of pivotal importance. A non-invasive, bedside monitoring modality for cellular oxygenation could provide direct information about oxygen transport. This allows clinician to adjust their management on actual measurements of tissue perfusion and oxygenation instead of systemic circulatory measures. In this light, monitoring of cellular oxygenation has been suggested to be beneficial during neonatal-cardiac surgery due to the highly affected hemodynamics (10). Yet, major non-cardiac congenital anomalies which requires surgery within the first days causes alterations in the neonatal physiology as well (4)(7). The recent introduction of the non-invasive Cellular Oxygen METabolism (COMET) monitor (Photonics Healthcare B.V., Utrecht, The Netherlands) makes it possible to measure in vivo mitochondrial oxygen tension (mitoPO_2). Although mitochondrial oxygen sensing has been recognized as a promising technique for pediatric ICU and anesthesia (11,12), until now reported use has been limited to adults (13–16). The present study tests feasibility and safety of intraoperative use of COMET monitoring in infants for the first time.

The COMET monitor measures mitoPO_2 by means of oxygen-dependent quenching of delayed fluorescence (17). Green pulsed laser excitation of protoporphyrin IX (PpIX) leads to a relatively long-lived red-light emission, called “delayed fluorescence”. The intensity of the delayed fluorescence decays with an oxygen-dependent lifetime, meaning more oxygen results in a shorter lifetime and vice versa. PpIX is the final precursor of heme

in the heme-biosynthetic pathway, synthesized inside the mitochondria. Under normal (non-sensitized) conditions PpIX concentrations in human skin are very low and non-detectable with COMET. Administration of 5-aminolevulinic acid (ALA) increases mitochondrial PpIX concentrations and ensures the mitochondrial origin of the delayed fluorescence signal (15). Therefore, to enable measurements with the COMET monitor, ALA needs to be applied on skin to induce PpIX, the latter acting as mitochondrially located oxygen-sensitive dye (17,18).

ALA is registered for use in adults, for example for photodynamic therapy in dermatologic pathology (19,20) and to visualize brain tumors during fluorescence-guided surgery (21,22) and was not used in pediatric patients until recently. Research with cutaneous ALA administration up to 354 mg in infants of 5 years and older reported no side effects.(23) Oral administration of 20 mg/kg ALA in infants of 1 year and older showed a transient increase of alanine aminotransferase (24)(25)(26). Rarely, the administration of 5-aminolevulinic acid led to an allergic reaction, in here contact dermatitis are the only reported allergies.(27) Therefore, we assumed the safety on a systemic level of a very low dosage of ALA – 8mg – on the skin of neonates, providing an opportunity to use COMET monitoring in neonates for the first time. Primary outcomes of this study were feasibility and safety, especially local (photo)toxicity, of cutaneous ALA administration in combination with using the COMET monitor in neonates perioperatively. A secondary outcome was preliminary evaluation of anesthesiologic and surgical procedures influencing mitoPO_2.

Material and Methods

The institutional research board approved a feasibility study of 15 neonates (MEC 2017-145).

After obtained informed consent from both parents, measurements were performed during surgical treatment of neonates with congenital diaphragmatic hernia (CDH) or esophageal atresia (EA). Surgery took place in the operating theater, unless the neonate was on extracorporeal membrane oxygenation (ECMO), in which case the surgery was performed in the pediatric intensive care unit due to logistics.

In this study the feasibility was defined as the possibility of priming the skin with ALA and to measure mitoPO_2 in neonates. The safety was defined as (the lack of) any adverse event of the skin after cutaneous administration of ALA and measurement with COMET until 48 hours after the COMET-skin sensor was removed.

An Alacare® plaster has a square format of 2 by 2 cm and contains 2mg per cm^2 ALA (Alacare, photonamic, Pinneberg, Germany). The plaster is covered by an aluminum layer to protect the primed skin to light exposure (Figure 1) (28). The plaster was applied in the pediatric intensive care unit (ambient temperature of approximately 22 degrees of Celsius) on the skin on the frontal side of the upper leg for at least 4 hours before starting the measurement. Research in adults showed that a priming time of 4 hours or more was needed to synthesize the suitable concentration of PpIX to enables measurements of mitoPO2 in the skin (15).The same minimal priming time was maintained in this study.

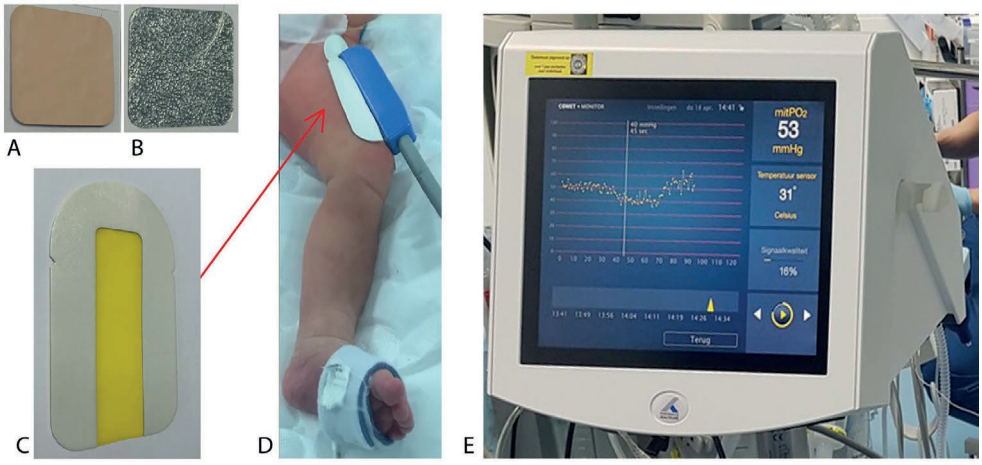

Fig 1. The ALA plaster with the aluminium cover (A) and the ALA side (B), double-sided tape (C) which is used for the application of the COMET-skin sensor (red arrow) on the frontal side of the upper leg (D) and the Cellular Oxygen METabolism (COMET) monitor (E).

The COMET-skin sensor has a biocompatible housing of 7 x 2 x 2 cm. The skin sensor was placed on the primed skin and was attached to the skin by a double-sided plaster provided by the COMET manufacturer (Figure 1). The influence of light on the primed skin during the application of the COMET-skin sensor was minimized by turning off the surgical luminaires/lamps. After the application of the skin sensor, the biocompatible housing was covered with aluminum foil.

Continuous registration of routine vital parameters, regional cerebral oxygenation (rSO_2) (INVOS™ 5100C) and mitochondrial saturation (COMET) were obtained and stored for off-line analyses. Sampling rate of the vital parameters was every second, rSO_2 every 6 seconds and mitochondrial oxygen tension ($mitoPO_2$) every 60 seconds. Intraoperative management was registered in our Patient Data Management System. Patients received

general anesthesia with sevoflurane/midazolam, rocuronium and fentanyl. MitoPO$_2$ measurements started before surgery and continued until after surgery. After completion of the measurement the primed skin was shielded against light with an aluminum plaster for 48 hours. This is based on the pharmacological characteristics of ALA. The mean half-life fluorescence clearance of PpIX is 30 ± 10 hours.

Results

Informed consent was obtained in 11 CDH and 4 EA patients. Intraoperative measurements were performed in all 15 included neonates. Neonates had a median gestational age of 38 weeks (IQR 37,7 – 40,2), a median birth weight of 3,000 grams (IQR 2,400-3,340) and a median age at surgery of 2 days (IQR 2-5.5). Median duration of the surgical procedure was 106 minutes (IQR 95-116) and two patients received surgical repair of CDH on ECMO in the pediatric intensive care unit (Table 1). Median skin priming time with ALA was 7h45m (IQR 6h50m – 12h0m). Twelve out of 15 measurements were successful with a median duration of the MitoPO$_2$ measurement of 116 minutes (IQR 98-133) (Table 1). The first measurement failed due to the radiant warmer (infra-red light), the second due to pink chlorohexidine-alcohol disinfectants and the third due to purple skin marker on the primed skin.

Table 1. Patient demographics

n=15	**Median (IQR)**
Male gender, n (%)	8 (53%)
Gestational age, wk	38.1 (37.7 – 40.2)
Birth weight, grams	3000 (2400 – 3340)
Age at surgery, days	2 (2 – 5.5)
Duration of surgery, min	106 (95-116)
Priming time skin, min	465 (413 – 720)
Duration MitoPO$_2$ measurement	116 (98 – 133)
Surgical approach	
Thoracoscopy, n (%)	5 (33%)
Thoracotomy, n (%)	2 (13%)
Laparotomy, n (%)	8 (53%)
Surgery during ECMO, n (%)	2 (13%)

In the 12 successful measurements (Table 2) the mitoPO$_2$ interquartile range at start of the measurement was 51 – 60 mmHg. In all neonates the skin was examined on regular timepoints; after removing the ALA plaster after priming of the skin, directly after removing the COMET-sensor, at 24 hours and 48 hours after removing the COMET-sensor. No adverse events such as erythema or other signs of an irritated skin were observed.

Table 2. Median and IQR values of the 12 successfully obtained measurements

	HR	*MABP*	*Saturation*	*rSO$_2$*	*MitoPO$_2$*
Start measurement	*133 (113 – 142)*	*41 (37 – 44)*	*96 (94 -97)*	*87 (66 – 93)*	*58 (51 – 60)*
+10 minutes	*130 (112 – 146)*	*48 (40 – 53)*	*94 (91 – 97)*	*83 (69 – 92)*	*57 (55 – 64)*
+20 minutes	*133 (118 – 140)*	*49 (40 – 62)*	*96 (93 – 97)*	*88 (69 – 93)*	*54 (53 – 63)*
+30 minutes	*133 (122 – 151)*	*47 (44 – 49)*	*95 (94 – 97)*	*81 (74 – 93)*	*53 (49 – 60)*
+40 minutes	*146 (135 – 160)*	*42 (35 – 46)*	*92 (90 – 97)*	*79 (70 – 88)*	*53 (52 – 56)*
+50 minutes	*144 (137 – 156)*	*41 (35 – 48)*	*95 (91 – 99)*	*82 (72 – 89)*	*50 (48 – 54)*
+60 minutes	*149 (137 – 164)*	*43 (39 – 45)*	*97 (91 – 99)*	*88 (77 – 95)*	*51 (49 – 54)*
+70 minutes	*154 (136 – 166)*	*45 (40 – 48)*	*96 (92 – 97)*	*87 (65 – 94)*	*52 (49 – 58)*
+80 minutes	*150 (137 – 168)*	*45 (35 – 46)*	*96 (95 – 99)*	*86 (71 – 95)*	*52 (47 – 59)*
+90 minutes	*151 (133 – 168)*	*42 (37 – 48)*	*97 (91 – 99)*	*83 (67 – 94)*	*53 (52 – 59)*
+100 minutes	*157 (124 – 163)*	*42 (39 – 45)*	*96 (92 – 99)*	*78 (65 – 91)*	*51 (50 – 63)*
+110 minutes	*133 (121 – 168)*	*46 (42 – 52)*	*97 (93 – 99)*	*84 (68 – 91)*	*53 (50 – 64)*
+120 minutes	*137 (127 – 171)*	*42 (37 – 52)*	*96 (92 – 99)*	*77 (74 – 91)*	*48 (45 – 53)*

Two cases illustrate fluctuations in mitoPO$_2$ in relation to surgical and anesthetic actions. Case 1 (figure 2A) is a female neonate, gestational age 37 weeks, birth weight 2,500 grams, with CDH requiring veno-arterial ECMO treatment due to therapy-resistant pulmonary hypertension. Surgical treatment was on day 8 of life, during ECMO. Priming of the skin with ALA was 6 hours. During surgery bleeding intercostal arteries caused significant blood loss. Vital parameters and rSO$_2$ remained unchanged, but mitoPO$_2$ decreased from 62mmHg at start surgery to 36mmHg (a reduction of 42%) during blood loss and partially recovered after supplementation with erythrocyte transfusion with a mitoPO$_2$ up to 53mmHg at the end of the surgery.

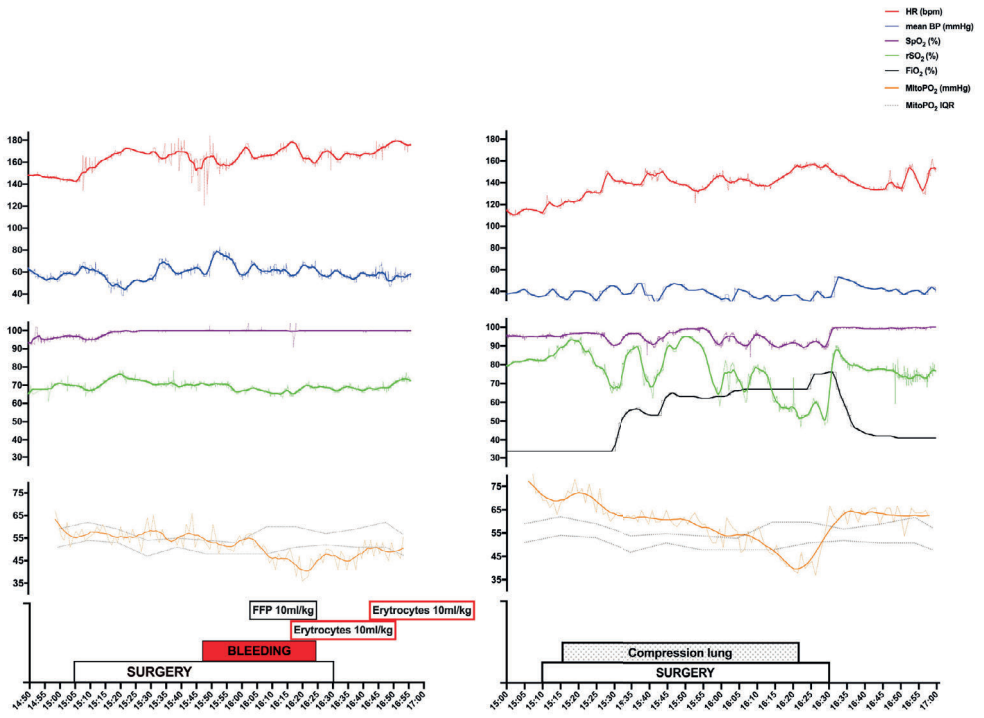

Fig 2. Surgical repair of congenital diaphragmatic hernia during EMCO (A) Surgical repair of esophageal atresia (B).

Case 2 (figure 2B) is a male neonate, gestational age 34 weeks, birth weight 1,950 grams, with EA type C with a trachea-esophageal fistula. Surgical repair took place on day 1 of life. Skin priming time with ALA was 8 hours. The patient was positioned on the left side during surgery. Surgical compression of the lung caused hypoxia which required increasing FiO_2 from 35% to 75% to maintain peripheral saturation between 90% and 95%. Blood pressure and heart rate remained stable, rSO_2 responded on the increased FiO_2 firstly, but $mitoPO_2$ decreased soon after the compression started and continues to decrease from 69 mmHg at start surgery to 37 mmHg (a reduction of 47%) and restored within minutes after manipulation of the lung was finished with a $mitoPO_2$ up to 62mmHg at the end of the surgery.

Discussion

This is the first study showing feasibility of mitoPO$_2$ measurements in neonates, and importantly, in a clinically relevant high-risk perioperative setting. Measurements with the COMET monitor proved feasible and safe in terms of local damage to the skin. Furthermore, pathophysiological disturbances led to perturbations in mitoPO$_2$. In twelve out of fifteen patients mitoPO$_2$ measurements were successful. Failures were caused by external and potentially preventable factors, disabling detection of an adequate delayed fluorescence signal. In one case infrared warming lamp heat or radiation interfered with the priming of the skin with ALA. Aluminum foil is a strong infrared reflector and was successfully used to shield the ALA plaster against infrared radiation during priming of the skin in the following cases. In the two other failed cases colored substances on the skin interfered with measurements, chlorohexidine with pink pigment and skin marker are both significant sources of delayed fluorescence and thereby potent disturbers of the mitochondrial PpIX light emission.

Safety of ALA administration with Alacare plasters was a major concern for the ethics committee due to the off-label use of ALA for measuring mitoPO$_2$ with the COMET. The reaction of the neonatal skin on ALA administration was unknown and consequently we only obtained approval to perform this feasibility and safety study. ALA makes the skin sensitive for light, consequently it is frequently used for photodynamic therapy in different sorts of dermatologic pathology. In children of five years and older, the administration of ALA up to 354 mg, which is over 40 times higher than de 8mg ALA that was applied on the skin in this study, did not have any side effects.(23) Oral administration of 20 mg/kg ALA in infants of 1 year and older showed a transient increase of alanine aminotransferase (24)(25)(26). Systemic effects of topical/local administration of ALA on the skin have not been reported and in this study, we focused on potential local side effects in neonatal skin.

There is a risk for erythema and burns when the skin is exposed to (day)light after the administration of ALA. Therefore, precautionary measures were taken to shield the skin for light for 48 hours after the measurement with the COMET was ended and the skin sensor was removed. In none of the cases local damage or irritation of the skin was observed, so the combination of ALA-plaster and COMET measurements seems safe.

The pharmacokinetic properties of topical ALA administration with Alacare in neonates are unknown, but in adults the reported skin priming time with ALA takes 4 till 8 hours (13). In this study, the same priming times were maintained for neonates. In a following

efficacy study, the power calculation/sample size will be focused on validating mitoPO$_2$ measurements in neonates and analyzing the ideal priming time of the neonatal skin. This will create insight in the reaction of the skin to the application of ALA in term and preterm neonates.

For this study two major non-cardiac congenital anomalies were included: congenital diaphragmatic hernia (CDH) and esophageal atresia (EA). These congenital anomalies were chosen to be eligible because major surgery is required within the first days of life and postoperative brain injury are reported in children with these congenital anomaly (4)(7). CDH neonates suffer from lung hypoplasia and abnormal morphology of the pulmonary vasculature which results in respiratory insufficiency and severe (therapy-resistant) pulmonary hypertension (29)(30). CDH neonates are a challenge for clinicians to manage due this altered physiology. In EA neonates, the physiology is less affected by the congenital anomaly itself, but requires complex surgery with major intrathoracic manipulation which highly affects the neonatal physiology (31). In these children, our preliminary results suggest that monitoring mitochondrial oxygenation might register changes in neonatal physiology which could not have been observed using standard monitoring devices. Clearly, further research into the clinical usability of COMET is warranted but seems justified based on this pilot. Although this was only a feasibility and safety study, these results confirm that mitochondrial hypoxia may occur without clear signs of central hypoxia and are in line with previous research in animals and humans (32–35). A piglets study demonstrated cutaneous mitoPO$_2$ changed earlier than MABP and lactate during ongoing hemodilution (32). In a sepsis rat model as well as in rats with induced right ventricular failure due to pulmonary arterial hypertension, mitoPO$_2$ proved an additional parameter monitoring physiological changes (33,34). The clinical prototype of the COMET was tested in healthy volunteers and showed measuring mitochondrial oxygenation and oxygen consumption in humans (13). Previous reports demonstrated the intraoperative use of COMET in adults (15) and also demonstrated that mitoPO$_2$ measurements are not limited to the skin (35). The first study using COMET during upper gastro-intestinal endoscopy showed it is technically feasible and safe (35).

Adequate oxygen supply to tissues is of pivotal importance to sustain mammalian life. Aerobic metabolism is maintained through inhalation of air in the lungs and subsequent transport of the absorbed oxygen to tissues via the circulation. The flow of hemoglobin-bound oxygen through the macro- and microcirculation and diffusion of molecular oxygen into the tissue cells brings oxygen to the mitochondria. In the mitochondria, oxygen is used in oxidative phosphorylation in order to efficiently produce adenosine triphosphate (ATP)

that acts as the energy source for many cellular processes. Furthermore, mitochondria are essential for homeostasis of the cell, they play a major role in (programmed) cell death (apoptosis). Opening of the mitochondrial permeability transition pore, as a result of a stressful stimulus such as calcium or reactive oxygen species overload, leads to loss of the mitochondrial membrane potential(36). The collapse of the membrane potential results in ATP depletion and necrosis(37), and the release of mitochondrial content such as cytochrome c leads to apoptosis(38). A correlation to outcome after perturbations in cellular oxygenation have not yet been shown, but it could be used as an early warning sign. Importantly, in both a preclinical (32) and clinical setting (15) mitoPO$_2$ provided different information than hemoglobin saturation-based techniques like near- infrared spectroscopy (NIRS). Although visible light spectroscopy and near-infrared spectroscopy failed to show any response on a perturbation, mitoPO$_2$ clearly dropped. This was observed during hemodilution in piglets, where mitoPO$_2$ was measured simultaneously with tissue oxygen saturation on the thoracic wall. The mitoPO$_2$ decreased after the hemoglobin dropped below a threshold, but tissue oxygen saturation, which was measured with NIRS, did not (32).

We previously published a clinical example in which mitoPO$_2$ showed a different response than microvascular hemoglobin-saturation. During peripheral vasoconstriction, which was induced by the administration of clonidine, microvascular flow and velocity parameters measured with laser-doppler decreased both. The venous-capillary oxygen saturation did not decrease, however, mitoPO$_2$ in the skin measured by COMET decreased along with the decrease in flow and velocity (15). While mitoPO$_2$ and microvascular flow provided similar information here, we expect additional value of mitoPO$_2$ measurements in clinical situations in which microvascular shunting (39) and loss of hemodynamic coherence occur (40), for example in sepsis and hemodilution. During sepsis microcirculatory dysfunction occurs which causes shunting and loss of the coherence between blood flow and tissue oxygenation. Here microvascular, and ultimately mitochondrial, oxygen measurements can be of additional value (39). The same holds true during a hyperdynamic circulation due to hemodilution, causing erythrocytes to pass too quickly through the microcirculation. This phenomenon is referred to as functional shunting and involves the inability of hemoglobin to off-load oxygen fast enough to the tissues as it passes through the microcirculation, causing cellular hypoxia while hemoglobin saturation is normal or increased (40)(41).

In this study we found baseline mitoPO$_2$ values in the range of 51-60 mmHg. In a previous study in healthy volunteers we reported mean mitoPO$_2$ to be 44 mmHg, and in a very recently published study in critical care patients mean mitoPO$_2$ was reported to be around 60 mmHg (42). Such relatively high values match well with other oxygen measurements in skin (43). The differences between the studies could well be attributed to factors like skin temperature, filling status of the patient, and use of sedation/anesthesia, since such factors are known to influence skin perfusion. Clinical data until now are scarce and normal values for mitoPO$_2$ remain to be determined, as well as the influence of patient factors (such as age) and clinical circumstances. Although we do think mitochondrial oxygen tension is in general higher than anticipated (12), the reader should realize that mitoPO$_2$ in other organs and tissues is likely to differ. Differences in tissue oxygen levels exist between organs, tissues and tissue compartments (43) and metabolic activity (for example muscle contraction) is also of influence.

To date, clinicians are in the dark about the effect of the altered neonatal (patho) physiology during major high-risk surgery on cellular oxygenation. In the past the focus was to optimize macrohemodynamics although the microcirculation has been increasingly recognized as an import variable in the critically ill neonate (44). To measure tissue oxygenation, a modality based on the principle of near infrared spectroscopy (NIRS) became popular. The optode of the NIRS emits near-infrared light, which easily penetrates biological tissue at a depth of approximately 2 to 3 cm.(45)(46) It measures the oxygenation of a combination of 75% venous, 20% arterial and 5% capillary blood, but does not provide information about the oxygen concentration at cellular level. Unfortunately, the clinical use of additional monitoring with NIRS have not been established yet.(47) The COMET allows us to look at oxygen availability at a cellular level. The neonatal skin is an ideal target organ for COMET measurements. It is the biggest organ in neonates and has a relative bigger surface and is more vascularized compared to adults. Skin blood circulation is very sensitive to changes in vascular resistance and blood pressure (48), potentially making the skin a good indicator for the (general) cardiopulmonary status of the neonate.

Compared to interstitial measurements with for example oxygen electrodes COMET has some distinct advantages, such as no need for calibration, non-destructiveness (no need for needle placement), well-defined measurement compartment and very fast response time (no need for signal integration over longer periods of time). A disadvantage of the COMET technique is the necessary priming with ALA. Although previous studies in adults and this study in neonates, show that with some precaution's application of ALA to the

skin can be done without harm, it requires planning and currently prevents its use in emergency situations. In elective situations in the operating room and for use in the intensive care this proved not a major issue.

In conclusion, this is the first study showing it is possible to measure mitoPO$_2$ in neonates and that the cutaneous administration of ALA to neonates in the described situation can be safely applied. Preliminary data suggests that mitoPO$_2$ in neonates responds to perturbations in physiological status. The added value of mitochondrial measurements for clinical decision making remains to be determined in future studies.

Conflict of Interest

Dr. E.G. Mik is founder and shareholder of Photonics Healthcare B.V., The company that developed and commercializes the COMET monitor. Photonics Healthcare B.V. holds the exclusive licenses to several patents regarding this technology, filed and owned by the Academic Medical Center in Amsterdam and the Erasmus University Medical Center Rotterdam, the Netherlands. The remaining authors declare that they have no competing interests.

Author Contributions

All authors had a substantial contribution to conception and design, and/or acquisition of data, and/or analysis and interpretation of data. All authors participated in drafting the article or revising it critically for important intellectual content and gave final approval of the version to be submitted.

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Chapter 10

Summary

Summary

In this thesis we described the development of the CE-marked COMET monitor to a bedside monitor of mitochondrial oxygenation and oxygen consumption. Earlier methods to measure mitochondrial function and cellular oxygenation at the bedside were invasive and difficult to perform. Measuring mitochondrial function in sepsis may prove to be fundamental for fully understanding the pathophysiology of sepsis. Proving the stability of the COMET monitor in sepsis like models in animals and healthy volunteers is a necessary step towards the clinical implementation of this monitoring technique.

In **chapter 2** we described mitochondrial function and its adaptation to the pathophysiology of critical illnesses. Adenosine triphosphate (ATP) production through the process of oxidative phosphorylation is the primary and best known function of mitochondria. Oxidative phosphorylation has a high affinity for oxygen and thus works under low levels of oxygen. Downregulation of mitochondrial function as a consequence of sustained low levels of oxygen is called oxygen conformance, and may last well past the initial exposure to low oxygen concentrations. Oxygen conformance, mitochondrial damage and mitochondrial hits may all work together to a state called cytopathic hypoxia. This is a state in which the delivery of oxygen to the cells is sufficient but cellular or mitochondrial metabolism is hampered. We believe that in the complex pathophysiology of critically ill patients a multilevel approach is required. The interaction between macro-circulation, micro-circulation, and parenchymal cells in critically ill patients is complex. Measuring mitochondrial function may provide fundamental insights into the complex pathophysiology of these patients.

We described the COMET monitor and its output in **chapter 3**. We observed a decline in cellular oxygen in this first use of the COMET monitor on a patient, which wasn't observed on the O2C monitor (the current standard method for observing tissue oxygenation). These results suggest the promising potential of the COMET monitor in detecting cellular hypo-oxygenation in critically-ill patients at the bedside.

We validated the earlier calibration of the COMET monitor in human skin in **chapter 4**. For the oxygen consumption kinetics we compared the COMET measurement with the LEA O2C, SenTec OxiVenT and Medtronic INVOS parameters during a vascular occlusion test. For the dynamic consumption measurement used in the COMET monitor not only vascular occlusion but also direct pressure on the site displacing oxygen carrying erythrocytes out of the microcirculation is of great importance. The COMET monitor's method to determine mitochondrial oxygen consumption differs in technique and

measures a faster and more profound decline of mitoPO$_2$ compared to the arterial occlusion test used with the other monitors.

With the new protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT), the technique behind the COMET monitor, *in vivo* determination of cellular oxygenation and oxygen consumption can be measured. In **chapter 5** the PpIX-TSLT technique to asses mitochondrial function is compared to the most commonly used *ex vivo* technique based on isolated mitochondria from muscle biopsies using a Clark-type oxygen electrode. In the endotoxemia model used in rat, which is based on infusion of lipopolysaccharide and thereby creating an sepsis like endotoxemia, we measured a decrease in mitochondrial function *in vivo* but not *ex vivo.*

Earlier studies suggested the diminutive effect of endotoxemia on mitochondrial function is predominantly by inhibition of complex 1 of the electron transporter chain. The electron transporter chain is the functional unit of the mitochondria responsible for ATP production, one of the essential functions of mitochondria for cellular homeostasis. The dominant electron pathway for the electron transporter chain is via complex 1 and NADH. We showed the expected decrease in mitochondrial function was prohibited by administration of succinate, an electron donor for complex 2 of the electron transporter chain. Showing the possibility of the PpIX-TSLT to measure decline and preservation of mitochondrial function in this endotoxemia model.

Using the same model of endotoxemia in human volunteers we used the COMET monitor to evaluate changes in mitoPO$_2$ and mitoVO$_2$ during the experiment as described in **chapter 6**. Although the LPS-induced endotoxemia was only mild in the healthy volunteers we measured a decline of mitoPO$_2$ directly after LPS administration. Interestingly the mitoVO$_2$ increased compared to the baseline measurement, showing a different pattern in this endotoxemia model. Measurement of these changes at bedside paves the way for monitoring of mitochondrial function in patients.

In **chapter 7** we described the interim analysis of our first study on the ICU using the COMET monitor. We measured mitochondrial function in ICU patients with sepsis at bedside using the COMET monitor. Off-site mitochondrial function was measured *ex vivo* in platelets and mitochondrial damage was assessed by determining mitochondrial DNA in plasma.

Another very dynamic and demanding environment for optical measurement is the operating theatre. In **chapter 8** we showed the first measurements of mitoPO$_2$ over time

during neurosurgery. The advantage of measuring in neurosurgery is the overall stability of the patients undergoing these operations and the duration of the procedure needed to perform neurosurgery. We were able to obtain stable measurements of mitochondrial oxygenation during stable operating conditions.

It was possible to monitor mitochondrial oxygenation in neonates during congenital esophageal atresia or diaphragmatic hernias repair operations, as described in **chapter 9**. Overall the technique did not cause any damage to the skin of the neonates and in 12 out of 15 cases intra operative monitoring was possible. In 3 of these 15 patients measurement of mitochondrial oxygenation was not possible, external and potentially preventable factors were at least partly responsible for failure of the monitoring technique. Exposition of the measurement site to infra-red light, a potent light source, caused depletion of the buildup PpIX. Purple skin marker or the disinfectant chlorohexidine causes excess delayed fluorescence and thereby failure to measure the mitochondrial source. Preliminary data showed the sensitivity of mitoPO_2 to the perturbations in physiological status caused by the operation or anesthesia. We showed it is possible to measure mitoPO_2 in neonates and its possible additional value next to the standard monitoring modalities used.

Chapter 11

General discussion

General discussion

In the work we presented in this thesis we have shown robustness and sensitivity of the COMET monitoring technique in our pre-clinical work. The main goal, measuring mitochondrial dysfunction in sepsis, was partly achieved in our ICU study, we failed to reach sufficient participants to analyze the data. We were able to measure cellular oxygenation in patients under anesthesia and started to show the additional value of the COMET monitor as a monitor of cellular oxygenation during anesthesia.

The electron transport chain and its production of adenosine triphosphate (ATP) is the best-known function of mitochondria. However, the role of mitochondria in homeostasis of the cell is not limited to the production of ATP. Mitochondria play a key-role in calcium metabolism and cell death mechanisms (**Chapter 2**). Measuring mitochondrial oxygenation *in vivo* at the bedside may help in determining the role of mitochondrial dysfunction in acute changes during severe illness [1]. We described the journey of the protoporphyrin IX triplet state life technique (PpIX-TSLT) from a laboratory setup to a CE-marked device for use at the bedside. We described the function of COMET monitor and its first use on a patient during neurosurgery in **chapter 3.**

The technique of the COMET monitor has extensively been calibrated in cells, organs and rats [2,3]. The calibration of the monitor in human skin could not be performed in the same way as in the preclinical work, given that complete deoxygenation of the site of measurement by nitrogen admission to healthy volunteers is not considered safe. Using direct pressure on the probe in combination with arterial occlusion of the arm provided the lowest measurable concentration of oxygen in the mitochondria. This approximation of the zero oxygen condition together with the combination of arterial puncture and blockage of mitochondrial respiration allowed for a two-point verification of the calibration constants in man. Measuring cellular oxygen consumption with the COMET monitor by the method of only arterial occlusion showed similar dynamics as the O2C, OxiVenT and INVOS parameters. The dynamic measurement of mitoVO_2 with direct pressure on the probe gave a faster and more pronounced reduction of mitoPO_2. We showed that earlier calibration in the lab was successfully translated to the COMET monitor **(chapter 4).**

In part two of this thesis we described the changes of mitochondrial oxygen consumption in a lipopolysaccharide(LPS)-induced endotoxemia rat model. Although we measured a decline of mitochondrial respiration *in vivo,* these changes were not matched by our *ex vivo* measurements of mitochondrial oxygen consumption (**chapter 5**). Measurement

of mitochondrial function in sepsis is notoriously difficult. Bedside monitoring was not available and *ex vivo* determination of mitochondrial function is difficult and laborious to perform. Moreover, ex vivo measurements are disruptive and alter the cellular environment. In critically ill patients an increase, decrease, or stable oxygen consumption by the mitochondria have been described. Recent work showed that the effects of the medium used in *ex vivo* tests play a major role in these tests [4,5]. Our findings in **chapter 5** underline this phenomenon in which *in vivo* mitochondrial dysfunction is measured but *ex vivo* tests with a lab-based medium failed to measure mitochondrial dysfunction. These observations could point to a possible reversal of mitochondrial dysfunction in this stage of change in our endotoxemia model.

LPS was also used in a human volunteer model to study the initiation phase of sepsis. In collaboration with the Radboud UMC we measured the effect of LPS on mitoPO$_2$ and mitoVO$_2$ in healthy volunteers (**chapter 6**). Most interestingly, we measured a direct decline of mitoPO$_2$ 1.45 hours after LPS administration, whereas no decline was observed in the control group. In the LPS group mitoVO$_2$ increased significantly over time versus no observed change in the control group. Measuring a change in mitoPO$_2$ but not in mitoVO$_2$ show these are distinct parameters which can change independently. Measuring a decrease of mitochondrial function in our rat model but an increase of mitochondrial function in our healthy volunteer model is an example of the difficulty in determining the role of mitochondrial dysfunction in the pathophysiology of sepsis.

The PpIX-TSLT technique is robust in the preclinical phase, given that mitochondrial function is required to produce the PpIX signal. However, measurement of mitochondrial parameters with the COMET monitor in patients with sepsis was not a certainty. Although we did not reach the first target number of occlusion in our ICU study, we were able to successfully use the COMET monitor on the ICU and complete various measurements (**chapter 7**). Recently, Coldewey et al. showed usability of COMET measurements in septic patients [6].

In part three we showed the ability to measure mitochondrial oxygenation during neurosurgery in the intraoperative setting in a challenging environment, with strong interference of the surgery lights on our signal (**chapter 8**). Measuring stable mitoPO$_2$ values over time when the clinical status is also stable shows a reliability of the monitoring technique under anesthesia. The COMET monitor also proved viable during neonatal surgery, as we presented in **chapter 9.** When the mitoPO$_2$ values change in patients with hemodynamic challenges during an operation, as shown in both chapters,

the monitoring technique might be of additional value for an anesthesiologist during challenging operations. The goal of an anesthesiologist, safeguarding oxygen supply and thereby organ function during an operation, may benefit from direct cellular oxygen monitoring.

Future perspectives

In our experimental work we should link the change of mitochondrial function *in vivo* (COMET) and *ex vivo* (mitochondrial function in platelets in own serum) to the elevation of mtDNA. Measuring the changes of *in vivo* and *ex vivo* mitochondrial dysfunction over time may show which method of monitoring mitochondrial function is more sensitive to measure these changes earlier. We should measure the effect of treatment of mitochondrial dysfunction on organ function or changes biomarkers associated with outcome like lactate concentration. Further work is needed to determine if a decline and elevation of mitochondrial function in sepsis is part of an pathophysiological pattern in sepsis or may even be a different phenotype of the disease.

The COMET monitor is sufficiently capable of measuring mitochondrial oxygenation and respiration on the ICU. There is no knowledge of normal values of mitochondrial respiration in the age group mostly affected by sepsis, namely the age group of 60 and over. Although finding a sufficiently linked control group to the sepsis group is challenging, I strongly believe the next studies with the COMET monitor should include at least an healthy control group and possibly also an IC control group.

As an example for an IC study I suggest measuring mitochondrial function as early as reasonably possible on the ICU in combination with a more longitudinal approach. In this longitudinal approach it would be nice to measure mitochondrial respiration multiple times per day for a couple of days. In the described longitudinal approach, measuring mitochondrial respiration during the different phases of sepsis will help with determining the role of mitochondria in the pathophysiology of sepsis.

The COMET monitor could be of additional value in the current field of cellular monitoring of oxygenation and mitochondrial function. At the moment we are finishing our ongoing studies of monitoring of cellular oxygenation in patients under anesthesia and using the decline of mitoPO$_2$ from baseline as a possible individual transfusion target. In the future monitoring of cellular oxygenation could help anesthesiologist during the stabilization of patients in major operations like multi trauma patients, transplantation of major organs or thoracic surgery.

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Chapter 12

Nederlandse samenvatting

Nederlands samenvatting

In dit proefschrift beschrijven we acht studies met als centrale vraagstelling hoe de CE-gemarkeerde COMET monitor gebruikt kan worden als een monitor waarmee mitochondriale oxygenatie en zuurstofverbruik gemeten kan worden aan het bed van de patiënt. Eerdere methodes om mitochondriale oxygenatie en functie te meten waren invasief, moeilijk om uit te voeren en niet geschikt om te gebruiken bij de IC patiënt met sepsis. Het meten van de mitochondriale functie in sepsis zou belangrijk kunnen zijn om de pathofysiologie van sepsis te doorgronden. Een noodzakelijke stap richting de klinische toepassing van de COMET monitor, is het aantonen van de stabiliteit van de monitor in sepsis achtige modellen in dier en gezonde vrijwilligers.

In **hoofdstuk 2** bespreken we de mitochondriale functie en haar aanpassing in de pathofysiologie tijdens sepsis. Adenosine trifosfaat (ATP) productie door oxidatieve fosforylering is de primaire en meest bekende functie van het mitochondrion. Oxidatieve fosforylering heeft een hoge affiniteit voor zuurstof en blijft dus werken met lage zuurstof concentraties. Het verminderen van de mitochondriale functie als gevolg van blijvend lage zuurstofconcentratie wordt zuurstofconformatie genoemd. Zuurstofconformatie kan voortduren lang nadat het initiële zuurstof tekort is hersteld. Zuurstofconformatie, mitochondriale schade en mitochondriale hits zouden samen kunnen werken, ook wel genaamd “cytopathic hypoxia”. Cytopathic hypoxia kenmerkt zich door voldoende zuurstof toevoer naar de cellen, maar een belemmering van het cellulair of mitochondriale metabolisme. Wij denken dat in de complexe pathofysiologie van kritisch zieke patiënten op meerdere niveaus naar zuurstof en metabolisme gekeken moet worden, dus niet alleen naar de variabelen op macro niveau, zoals bloeddruk en saturatie, maar ook op micro vasculair en cellulair niveau. De wisselwerking tussen macrocirculatie, microcirculatie en parenchymcellen in kritisch zieke patiënten is complex. Het meten van de mitochondriale functie zou fundamenteel inzicht kunnen geven in de complexe pathofysiologische veranderingen in deze patiënten.

We beschrijven de kenmerken van de COMET monitor en zijn variabelen in **hoofdstuk 3.** In het eerste klinische gebruik van de COMET monitor bij een patiënt observeerden we na toediening van een potente vasoconstrictor een afname van mitochondriale zuurstof welke niet werd gemeten door de O2C monitor (de huidige standaard voor het meten van weefsel oxygenatie). Het resultaat suggereert potentie voor de COMET monitor om mitochondriale hypo-oxygenatie te detecteren bij kritisch zieke patiënten aan het bed.

In **hoofdstuk 4** valideren we de eerder verkregen kalibratie van de COMET monitor op menselijke huid. Voor de zuurstofconsumptie kinetiek vergeleken we de COMET meting met de LEA O2C, SenTec OxiVenT en Medtronic INVOS metingen tijdens een vasculaire occlusietest. Voor de dynamische consumptiemeting met de COMET monitor is naast vasculaire occlusie ook de directe druk op de plek van de meting van belang. Door de directe druk op de plek van de meting verplaatsen de zuurstofdragende erythrocyten zich uit de microcirculatie. De methode die gebruikt wordt door de COMET monitor om mitochondriale zuurstof consumptie te meten verschilt niet alleen in techniek, maar ook in reactiesnelheid van de arteriële occlusietest die gebruikt wordt met de andere monitoren. Dit toont aan dat de zuurstofconsumptie meting van de COMET monitor minder afhankelijk is van variabelen in de microcirculatie, zoals hemoglobine concentratie en saturatie, en daardoor een reproduceerbare meting geeft.

Met de nieuwe protoporphyrin IX-triplet state lifetime techniek (PpIX-TSLT), de techniek van de COMET monitor, is *in vivo* bepaling van mitochondriale oxygenatie en zuurstofconsumptie mogelijk. In **hoofdstuk 5** vergelijken we de PpIX-TSLT om mitochondriale functie te meten met de meest gebruikte *ex vivo* techniek, die gebaseerd is op geïsoleerde mitochondriën van spierbiopten. In het gebruikte endotoxemiemodel bij ratten, dat een sepsisachtig beeld creëert door infusie van een lipopolysacharide, maten we een afname van mitochondriale functie *in vivo* maar niet *ex vivo.*

Eerdere studies suggereerden dat het remmend effect van de endotoxemie op mitochondriale functie vooral op complex 1 van de elektronentransportketen aangreep. De elektronentransportketen is het functionele deel van een mitochondrion en is verantwoordelijk voor ATP productie, een van de essentiële functies van de mitochondriën en nodig voor cellulaire homeostase. De dominante route voor elektronen via deze keten loopt via complex 1 en NADH. We hebben laten zien dat de te verwachte afname in mitochondriale functie tegengegaan werd door infusie van succinaat, een elektrondonor voor complex 2 van de elektronentransportketen. Hiermee hebben we aangetoond dat de PpIX-TSLT afname en behoud van mitochondriale functie kan meten in dit endotoxemiemodel.

Hoofdstuk 6 beschrijft hoe we met hetzelfde endotoxemiemodel bij gezonde vrijwilligers de mitochondriale oxygenatie en functie in het lichaam gemeten hebben met de COMET monitor. Alhoewel het LPS geïnduceerde endotexemie mild was in deze gezonde vrijwilligers maten we een directe afname van mitochondriale oxygenatie na LPS toediening. Het mitochondriale zuurstofverbruik nam toe over de tijd. Een duidelijk

verschil met de afname na LPS die we in ratten hebben gemeten in hoofdstuk 5. Mogelijk leidt een minder heftige endotoxemie eerder tot toename van mitochondriaal zuurstof verbruik. Doordat veranderingen in mitochondriale oxygenatie en functie aan het bed bij gezonde vrijwilligers gemeten kan worden, kunnen we deze parameters nu ook in patiënten gaan meten.

Hoofdstuk 7 beschrijft de interim analyse van onze eerste intensive care studie met de COMET monitor. We hebben de mitochondriale functie van IC patiënten direct aan het bed gemeten met de COMET monitor. In het laboratorium werd *ex vivo* mitochondriale functie bepaald in de bloedplaatjes en de schade aan mitochondriën werd gemeten door de bepaling van mitochondriaal DNA in plasma. Helaas is het ons niet gelukt om voldoende patiënten te includeren en konden we geen conclusies te trekken op grond van de beschikbare data.

Hoofdstuk 8 gaat in op de eerste metingen van mitochondriale oxygenatie tijdens neurochirurgie. De operatiekamer is een uitdagende omgeving voor optische metingen door de beperkte ruimte die beschikbaar is rondom de patiënt en de vervuiling van het optische signaal door de chirurgische lampen. De voordelen van het uitvoeren van deze metingen tijdens neurochirurgie is de stabiliteit van de patiënt en de relatief lange duur van de neurochirurgische procedures. We hebben stabiele metingen laten zien tijdens stabiele operatieomstandigheden. Dit betekent dat je kan verwachten dat onder stabiele omstandigheden geen grote variaties in cellulaire oxygenatie zijn te verwachten. Een snelle daling van mitochondriale oxygenatie kan dus wijzen op een probleem bij de patiënt.

Hoofdstuk 9 laat zien dat het mogelijk is om mitochondriale oxygenatie te meten tijdens operaties bij neonaten. De methode veroorzaakt geen schade aan de huid van de neonaten en bij twaalf van de vijftien patiënten was het mogelijk om tijdens de operatie oxygenatie van de mitochondriën te meten. Bij de drie patiënten waarin de meting niet lukte, waren externe en potentieel vermijdbare oorzaken tenminste deels verantwoordelijk voor het mislukken van de meting. Het blootstellen van de voorbereide huid aan infraroodlicht, een potente lichtbron die gebruik wordt om de neonaat warm te houden, zorgt voor depletie van de opgebouwde PpIX. Bovendien zijn de gebruikte markeerstift en ontsmetting door chloorhexidine een bron voor vertraagde fluorescentie, waardoor het zuurstof afhankelijke signaal verstoord wordt en de meting onmogelijk is.

De eerste data lieten zien dat mitochondriale oxygenatie snel reageerde op veranderingen in de neonaat die veroorzaakt werden door de operatie of door de anesthesie. In

deze studie hebben we laten zien dat het mogelijk is om mitochondriale oxygenatie peroperatief te monitoren en dat het mogelijk van toegevoegde waarde is boven op de standaard monitoring die nu veelal gebruikt wordt.

Chapter 13

Dankwoord

Dankwoord

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Chapter 14

Curriculum Vitae

Curriculum Vitae

Mark Alexander Wefers Bettink werd op 11 augustus 1985 geboren te Groningen. Na het behalen van het VWO diploma aan het Rijnlands Lyceum Oegstgeest en een korte studie periode in Delft in de maritieme techniek begon Mark in 2004 met de studie Bio Farmaceutische Wetenschappen aan de Leidse universiteit. In 2007 begon Mark met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens het afronden van de studie Geneeskunde is er begonnen aan het eerste onderzoek van dit proefschrift bij de afdeling experimentele anesthesie. De opleiding tot Anesthesioloog pijnspecialist, met als opleider prof. dr. R.J. Stolker, in het Erasmus Medisch Centrum is een jaar onderbroken geweest waarin een groot deel van het werk aan dit proefschrift uitgevoerd is. Sinds het begin van 2020 is Mark werkzaam in het Franciscus Gasthuis en Vlietland als Anesthesioloog en Pijnspecialist. In deze periode is de laatste hand gelegd aan dit proefschrift. Samen met zijn partner Eva Hoftijzer heeft Mark twee dochters, Jette en Yfke.