Summary of changes made to the original protocol listed

Original protocol:

Research protocol – v2.4 31-03-2017

Changes leading to protocol v2.5 28-06-2017:

- Not having a central venous catheter in situ has been dismissed as an exclusion criterium for the pilot study, since it will not affect the feasibility of the mitochondrial oxygenation measurement.

Changes leading to protocol v3.0 23-01-2018:

- Study group information updated and another member of the study group added to the protocol.
- Correction of spelling and grammatical errors.
- Addition of insufficient comprehensibility of the Dutch language as an exclusion criterium, since informed consent procedure is in Dutch.
- Changes made to the study design due to logistical problems encountered during the pilot study
    - We saw that inclusions were missed in patients receiving red blood cell transfusion in the first hours after cardiothoracic surgery, due to not being able to asked informed consent to the still sedated patient. Therefore, informed consent to cardiothoracic patients, which will be admitted to the ICU post-surgery, is asked 1 day before the surgery takes place.
    - Due to heterogeneity in red blood cell transfusion time, it has now been protocolized that red blood cell transfusions will be given in one hour. The clinician can still deviated from this time frame is the condition of the patient does not permit a one hour lead time.
    - During the pilot study, the investigators noticed missing information regarding smoking habit and New York Heart Association in almost all patients due to no standardized recording of these data.
    - There were 8 measurement moments during the pilot study in the 24 hours following red blood cell transfusion. Due to logistics and stability of the measurement during certain time points, the measurement moments were reduced to 6 measurement moments. The dismissed time points were 15 minutes after the end of red blood cell transfusion and 120 minutes after the end of red blood cell transfusion.
    - No IN vivo optical spectroscopy of cerebral oxygenation device can be used during the main study due to logistical problems. This measurement has been deleted from the protocol.
    - Sample size calculation is updated with the results of the pilot study.
    - The monitor of the study is specified and the monitorplan is updated.

Changes leading to protocol v4.0 19-10-2018:

- Not having a central venous catheter has been dismissed as an exclusion criterium in the main study as well due to low inclusion rate.

- Due to the slow inclusion rate the informed consent procedure has been updated. Now, all ICU patients admitted to the ICU and expected to stay at least 24 hours in the ICU are asked for participation in the study. If the patient is not able to give her/his consent, the legal representative will be asked.

Changes leading to protocol v5.0 27-02-2019:

- Due to the variable time frame between admission to the ICU and being able to do informed consent with the patient and/or their legal representative, possible research subjects were missed. Therefore, the informed consent procedure was optimized. If the patient or legal representative could not have been reached in time for written informed consent, while the patient met the eligibility criteria, the patient was included with deferred consent procedure. Informed consent was sought within 48 hours after inclusion of the patient.
- The protocol has been updated to the latest regulations regarding privacy (General Data Protection Regulation).

INsufficient cellular OXygen in ICU patients with anaemia:

the INOX ICU–2 study

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TITLE: Insufficient cellular oxygen in ICU patients with anaemia: the INOX ICU-2 Study

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Short title	Insufficient cellular oxygen in ICU patients with anaemia: the INOX ICU-2 Study
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Summary

Rationale: Evidence is increasing that in some cases a haemoglobin trigger of 7-8g/dl may be too low and that an individualized red cell transfusion strategy may benefit critically ill patients. New studies have shown the potential of a protoporphyrin IX-triple state lifetime technique to measure mitochondrial oxygenation tension (mitoPO2) in vivo, which possibly is an early indicator of oxygen disbalance in the cell and therefore a physiological trigger for red cell transfusion.
Objectives: 1. To determine the feasibility of using mitoPO2 and the variability of mitoPO2 measurements in critically ill intensive care unit (ICU) patients who are about to receive a red cell transfusion. 2. To describe the effects of red cell transfusion and the associated change in haemoglobin on mitoPO2 and on other physiologic measures of tissue oxygenation and oxygen balance 3.To describe the association between mitoPO2 and vital organ functions and clinical outcomes.
Study design: pilot study followed by a prospective multicentre cohort study
Study population: ICU patients with anaemia in whom a red cell transfusion is planned.
Main study endpoints:
Primary endpoint:

- Variability of mitoPO2 before and after red cell transfusion. This will be compared to traditional parameters used to measure tissue oxygenation and oxygen balance

Secondary endpoints:

- Association of mitoPO2 with (ischemic) organ damage
- Association of mitoPO2 with the microcirculation
- Duration of mechanical ventilation
- Need for continuous renal replacement therapy (CRRT)
- Length of ICU-stay
- Length of hospital-stay
- ICU-mortality
- 90-day mortality

Study procedure: Included patients will undergo red cell transfusion as planned. However, red cell transfusion will be delayed for 2 hours. At multiple predefined moments, before and after red cell transfusion, data collection including blood samples and measurements of mitoPO2 will take place. It will be directly processed.
Nature and extent of the burden and risks associated with participation, benefit and group relatedness: The risks are moderate in this study with no SAE known. The burden for the ICU-patients is moderate since it's a non-invasive measurement. The normal clinical practice will continue and will not be altered. Since most ICU-patients have in this very controlled environment a central venous catheter and arterial catheter in situ, they are the most ideal patients to investigate mitoPO2 feasibility, accuracy and associations with other physiological parameters.

1. Introduction and rationale

Depending on the duration of critical illness eventually all critically ill patients develop anaemia which may severely affect their recuperation.[1] Deleterious effects of severe anaemia include a generalized decrease in oxygen carrying capacity and potentially ensuing multi-organ failure. Whereas in the old days clinicians tended to transfuse red cells as soon as haemoglobin(Hb) levels were slightly below normal, this has radically changed since 1999. In 1999 the landmark TRICC trial showed that critically ill patients that received red cell transfusions at a Hb of 9 g/dl were not better off than patients that received red cell transfusions only after their Hb reached 7 g/dl.[2] The notion that critically ill patients with Hb above 7 or 8 g/dl, might not profit from red cell transfusions has been confirmed by other studies and is now widely adopted in clinical guidelines.[3-5] Yet, there is also increasing evidence that in some cases a Hb transfusion trigger of 7- 8 g/dl may be too low. Patients with an ischemic brain, spinal cord, or myocardium, with renal insufficiency, or older patients might thus profit from red cell transfusion at higher Hb levels.[6,7] Whether these findings are solid enough to change clinical practice is heavily debated.[8,9] These uncertainties about efficacy and safety of transfusion lead to undesirable ambiguities in ICU transfusion practice.

Mik et al. has recently introduced the protoporphyrin IX-triplet state lifetime technique as the first method to measure mitochondrial oxygen tension (mitoPO2) in living cells and tissues.[10] Experimental results have shown mitoPO2's robustness and clinical potential. [10-13] Animal research has shown that: (i) cutaneous mitoPO2 decreases abruptly during haemodilution;(ii) abrupt decrease in mitoPO2 precedes lactate increase (as a consequence of a lack of tissue oxygenation) and hemodynamic instability; (iii) abrupt decrease in mitoPO2 occurs at different (low) Hb for different individuals; (iv) cutaneous mitoPO2 could be a marker for the physiological limits of isovolemic haemodilution; (v) cutaneous mitoPO2 could be an indicator for the need of a red cell transfusion; (vi) if haemodilution is stopped after a predefined drop in mitoPO2, mitoPO2 remains stable; (vii) but after 2 hours kidney failure occurs and lactate rises; (viii) if instead a red cell transfusion is administered after this drop, mitoPO2 rises again and levels of lactate and organ functions remain normal.[13-15] Thus, mitoPO2 was able to predict the need for a red cell transfusion.

In the ongoing INOX ICU-1 study, a decision tool for red blood cell transfusion is being developed in critically ill patients using retrospective ICU data. The proposed INOX ICU-2 project is a phase 1 diagnostic study examining whether mitoPO2, a recently developed test measuring mitochondrial oxygen, is a potentially useful candidate to improve the INOX ICU prediction tool. The results of the INOX ICU-2 will reveal whether mitoPO2 could be used to further tailor red cell transfusion strategies to the individual patient and ultimately reduce the harmful effects of both under- and over-transfusion in ICU patients.

This is the very first research on mitoPO2's clinical applicability in critically ill patients with anaemia and the first attempt to personalize red cell transfusion decisions in the ICU based on individual

assessment of the oxygen balance on a mitochondrial level, i.e. the ability of oxygen delivery to tissues to fulfil actual oxygen demand. We hypothesize that mitoPO2 can also be used as an early predictor of organ dysfunction. If this is true, mitoPO2 might be used to administer or postpone red cell transfusion in patients with anaemia.

The results of the proposed project cannot be immediately translated to clinical practice. Using these results, we will design a phase 2 diagnostic study, most probably a randomized clinical trial that will yield applied knowledge with respect to personalizing red cell transfusion. Application will be in ICU patients with anaemia who might or might not profit from red cell transfusions. It will lead to a reduction of both over- and under- transfusion.

2. Objectives

2.1 General

The ultimate aim of the INOX ICU project is to tailor red cell transfusion strategies to individual ICU patients with anaemia with respect to the potential risks and benefits. After the proposed project we will know the essential test characteristics needed to ultimately assess the added value of the mitoPO2 for the transfusion decision. With these results it will be possible to proceed to the next phase. In the next phase (not part of this project) we will compare whether clinical outcomes of patients in whom transfusion strategies are determined using mitoPO2 results are better than clinical outcomes of patients in whom transfusion strategies are determined without mitoPO2.

2.2 Endpoints

In this phase 1 diagnostic study we aim to investigate mitoPO2 test's feasibility, accuracy, and associations with other physiological parameters of oxygen balance and tissue oxygenation and with ischemic organ damage in critically ill patients with anaemia.

Specific objectives:
1. To determine the feasibility and variability of mitoPO2 measurement in critically ill ICU patients before and after a red cell transfusion.
2. To describe the effects of red cell transfusion and the associated change in Hb on mitoPO2 and on other physiologic measures of tissue oxygenation and oxygen balance such as, arterial oxygen saturation, central venous oxygen saturation (ScvO2, measured in either the jugular or subclavian vein, if applicable), lactate, and DO2 minus VO2 (difference between oxygen consumption and delivery).
3. To describe the association between mitoPO2 and vital organ functions, as assessed by SOFA (Sepsis related Organ Failure Assessment) score, duration of mechanical ventilation, duration of vasopressor therapy, myocardial ischemia, serum creatinine, RIFLE classification for renal function, need for renal replacement therapy, Glasgow coma score, RASS, ICDSC score.
4. To describe the association between mitoPO2 measurements and the microcirculation

With this in mind, we will assess the following parameters:

1. Prognostic: duration of mechanical ventilation, need for renal replacement therapy, ICU-stay, hospital stay, ICU-mortality, 90-day mortality, measurements of ischemic damage (particular in patients following transfusion).
2. Etiologic: levels and time course of markers of ischemia-reperfusion in relation to the clinical course (such as duration of mechanical ventilation, need for renal replacement therapy, ICU-stay, hospital-stay, ICU-mortality, 90-day mortality, measurements of ischemic damage) particular in patients following transfusion.

3. Methodologic: the value of mitoPO2 in patients receiving red cell transfusion compared to markers of hypoperfusion and oxygen balance used in standard critical care: lactate, ScvO2, arterial saturation (SaO2), arterial oxygen tension (PaO2), venous oxygen tension (PvO2), cardiac index (CI)).

3. Design and population

3.1 Study design

First, we performed a pilot study in the LUMC among 20 ICU patients with anaemia in whom a red cell transfusion was administered. After assessment of the feasibility of the mitoPO2 measurement and sample size, we will now continue with a prospective cohort study. This study will be performed in the ICUs of the Leiden University Medical Center (dr. M.S. Arbous), the Amsterdam Medical Center (Prof. dr N.P. Juffermans and the Erasmus University Medical Center (dr E.G. Mik) on patients with anaemia in whom an arterial catheter is already in place. MitoPO2 and other parameters will be measured at inclusion, before red cell transfusion, and at various time points after red cell transfusion. Appendix II presents study design, measurements and outcomes for the pilot study, as well as the multicentre prospective cohort study. During the study nurses and clinicians will be blinded for the results of the mitoPO2, since the investigator executes the mitoPO2 measurement and will not reveal the results.

3.2. Study population

Inclusion criteria: All adult patients admitted to the ICU with a Hb below 6.3 mmol/l, who were planned to undergo a transfusion of red blood cells were included in the pilot study. In the prospective multicentre cohort study, only patients with a Hb below 6.3 mmol/l in whom an arterial catheter is in place and in whom a red cell transfusion is planned will be included.

Exclusion criteria:

-patients without a legal representative to ask for informed consent
-patients less than 18 years old
-pregnant or breast feeding women since there is no adequate data from the use of ALA in pregnant or breast feeding women[16]
-patients in need of emergency red cell transfusion e.g. bleeding patients
-patients with porphyria and/or known photodermatosis
-patients with hypersensitivity to the active substance or to the plaster material of ALA
-patients with an expected ICU stay <24 hours
-insufficient comprehensibility of the Dutch language

4. Treatment of subjects

Investigational product: PpIX-TSLT with Comet

The COMET's non-invasive cutaneous mitoPO$_2$ measurements rely on the PpIX-TSLT(protoporphyrin IX-triple state lifetime technique). The PpIX-TSLT technique measures oxygen by oxygen-dependent quenching of delayed fluorescence lifetime of ALA (5-aminovulinic acid)-induced mitochondrial PpIX. PpIX is the final precursor of heme in the heme biosynthesis pathway and is synthetized in the mitochondria. The conversion of PpIX to haeme is the rate-limiting step and therefore causes a weak delayed fluorescence lifetime. Administration of exogenous ALA enhances PpIX to detectable levels and enhances mitochondrial origin of the delayed fluorescence signal.[10] Measurements are based on the detection of time of extinction of red light emitted by the tissue following excitation with green light (figure 1). This technique was first published in 2006.[17] The technique has been tested and calibrated for use in isolated organs and in vivo.[18-20] The PpIX-TSLT technique is described in detail by Mik (2013).[10]

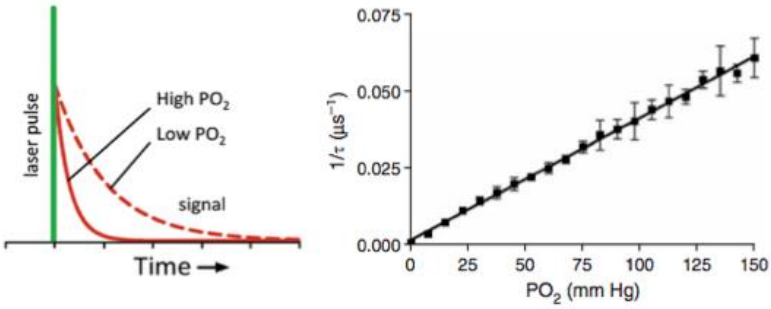

Figure 1: Detected delayed fluorescence. A short lifetime corresponds with high intra cellular oxygen concentration and low PO2 values correspond with a long lifetime (Mik, 2013). The lifetime can be translated to an oxygen tension with the help of a calibration

The COMET measurement system is an oxygen availability measurement tool that measures oxygen tension at the mitochondrial level by means of delayed fluorescence of protoporphyrin IX. The COMET monitor is used observationally. It will not be used to treat patients, nor is the information provided by the COMET used to alter patient treatment. The COMET measurement system is a non-invasive measuring system to determine cellular oxygen availability locally in human skin cells with a high concentration of protoporphyrin IX. It is CE marked as a medical device (DEKRA certificate number 2183975CE01, initially issued on 18 April 2016).

In this study, the mitoPO2 of the skin will be measured. Reliable measurements can only take place with sufficient concentrations of protoporphyrin IX in the mitochondria. Therefore, a self-adhesive patch containing 8mg of ALA (Alacare®; Spirig AG, Egerkingen, Switzerland) will be placed on the anterior chest wall for induction of PpIX, for at least 4 hours before the first measurement can take place. To enhance ALA penetration, the skin will be rubbed with alcohol to remove the exposed layers of stratum corneum. Hair, if present, will be shaved.[21] Following at least 4 hours induction of PpIX, the ALA patch will be removed after the outline of the ALA patch is marked. To create steady and reliable

measurements, a specially designed plaster will be used to attach the COMET Skin Sensor (COMET Skin Sensor, Photonics Healthcare, the Netherland) to the skin. This plaster is referred to as the Skin Pad, it is not CE marked, and is adhesive on both sides. One side of the plaster is a silicon skin friendly side and the other a strong acrylic adhesive to which the Skin Sensor is attached.

Figure 2: Skin Pad

After placing the skin sensor, calibration measurements will be made to ensure the quality of the measurements. Thereto, 5 measurements will be made in the first minute to establish a reliable and stable measurement. Occlusion of the microcirculation will be accomplished via local pressure with the measurement probe, which we use to validate the actual mitoPO2 measurement. After these measurements, mitoPO2 will be a measured each minute for 5 minutes to obtain a mean mitoPO2. To ensure that no other variables influence the measurements, measurement characteristics will be logged. In this log, the circumstances in which each measurement is made will be documented. Known intervening variables include, for example, skin temperature, thus skin temperature will be recorded. Other variables recorded in the log will be vasopressor use, dosage of the vasopressor, and nurse actions. Following mitoPO2 measurement, the ALA patch will be re-used to protect the exposed skin for phototoxicity until the next measurement. After completion of the mitoPO2 measurements the exposed skin will be protected from sunlight for 24 hours. An overview of the mitoPO2 measurement is given below.

The skin sensor and other parts of the COMET will be cleaned according to cleaning guidelines for medical devices in the intensive care unit. In this case, the COMET will be cleaned with 70% ethanol. In the case of visible dirt, the COMET will be cleaned with water and soap prior to ethanol 70% application.

Use of co-intervention

Due to the risk of phototoxicity, it is important for the skin exposed to the ALA patch to be protected from sunlight during, between, and for 24 hours following measurements.

Escape medication

Should adverse effects take place, the skin would need to be protected from further damage by light exposure. Possible adverse effects include burning sensation of the skin, pruritis, hyperpigmentation, application site erythema, and local pain.[22,23] Pain killers could be administered, if local pain would persist despite these precautions.

5. Methods

5.1 Study procedures

5.1.1 General

Since research has shown that approximately 10% of critically ill patients can make autonomous decision regarding research participation, most decisions will be made by their substitute decision makers, who are already overwhelmed by the medical information of the patient itself. Furthermore, studies have shown that obtaining contact with their substitute decision makers can be difficult in 7%, no decision maker exist in 6% of the cases. In addition, approximately half of the opportunities to recruit patients are missed because of workload and narrow time windows for inclusion. Difficulties in recruitment of eligible patient can lead to delayed identification of (in)effective treatment/diagnostics and limitation of generalizability. Alternative consent models are therefore needed to improve recruitment and minimize withdrawal.[24,25] Therefore, we will first ask all ICU patients admitted to the ICU, or their legal representatives, expected to stay at the ICU for longer than 24 hours for participation in our study. Patients admitted to the cardiothoracic department which will be admitted to the ICU post-operatively, will be asked for an informed consent on the day before the cardiothoracic operation. An information leaflet will be given and informed consent will be sought. Patients or their legal representatives will be told that a person can only participate in the study if he/she meets the inclusion criteria. However, if no legal representative could have been reached in person for written informed consent, while the patient meet the eligibility criteria, the patient will be included in the study and deferred informed consent will be sought within 48 hours after oral consent has been given. If written informed consent is available or oral consent given, at the moment the decision is made to administer a red cell transfusion, the self-adhesive ALA-patch will be placed. Due to logistics, the normal time elapsing between the decision of a non-emergency red cell transfusion and the actual administration is 2 to 3 hours. The induction time of PpIX is at least 4 hours, therefore red cell transfusion will be delayed by 1 to 2 hours. At no time red cell transfusion will be delayed in order to have enough time to induce PpIX if the patient is clinically in need of a red cell transfusion. To ensure this, the clinician responsible for the patient, and not the researcher, has the final decision in the timing of the red cell transfusion.

After inclusion of a patient meeting the eligibility criteria, each patient will be assigned an unique study number.

To determine the reliability of the mitoPO2 measurement in critically ill ICU patients with anaemia who will receive transfusion, we first assessed the variance of mitoPO2 from repeated mitoPO2 values before and after transfusion in 20 ICU patients. Next, using this variance, we estimated the number of patients needed to perform a reliable multicentre study[26], which we subsequently will perform.

To examine the effects of red cell transfusion and the associated change in Hb on mitoPO2, and on other physiologic measures of tissue oxygenation and oxygen balance such as DO2, VO2, ScvO2,

and lactate, we will compare values of all parameters before with the values after red cell transfusion. In addition we will perform similar analyses in subgroups of Hb and reason of admission to assess whether the observed effect of red cell transfusion on the parameters differs between the groups. We will also examine whether the decrease of the mitoPO2 precedes decreases of other measures of tissue oxygenation and microcirculation.

5.1.2 Clinical Care

It is standard clinical practice that most patients receive a central venous (vena jugularis, incidentally vena subclavia) and an arterial (arteria radialis) catheter when admitted to the intensive care unit. Patients with anaemia having an arterial catheter will be included in the study. During the pilot phase of the study, patients did not need to have a central venous catheter since the pilot study is about the feasibility of the mitoPO2 measurement and not the outcomes. Blood samples will be drawn from these catheters. Patient management will take place according to usual care. When patients meet the inclusion criteria, an ALA-patch will be placed. After at least four hours PpIX induction, the cutaneous mitoPO2 sensor will be placed to make the first measurements. Transfusion will be given in an one hour. The clinician can deviate from this time frame if the condition of the patient doesn't permit a 1 hour lead time. After red blood cell transfusion, measurements of the mitoPO2 will be made at predefined moments.

5.2 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons. These subjects will not be subjected to follow-up.

5.3 Data collection

5.3.1 MitoPO2 measurements

MitoPO2 is measured at the skin at the predefined moments described in detail in the next paragraph. With each measurement, the circumstances of the measurement will be recorded in the log (e.g. skin temperature) to assess possible bias of measurements.

5.3.2 Gathering and handling of clinical data

While waiting for the induction of PpIX after placement of the ALA patch, data will we gathered. Appendix 2 shows an overview of the data collection. First of all, demographic data will be collected: age in years, gender, weight, height, Body Mass Index, allergies, medication use, comorbidities (acute and chronic), and reason of ICU admission. Also, pulmonary function, renal function, APACHE-II and APACHE-IV will be assessed. The demographic data can be obtained from the hospital's electronic patient dossier system (EPD).

Clinical data will be assessed as well, which are: heart frequency (HF), blood pressure (BP), central venous pressure (CVP), and peripheral oxygen saturation (SpO2). These will be assessed at the same time points as the mitoPO2 measurements. Daily the Glasgow coma scale, 24h-urine production, electrocardiogram (ECG), RIFLE classification, SOFA score, RASS, and ICDSC score will be collected. These measurements are already done, as part of the daily intensive care management, and will be collected using the EPD. Other data collected from the EPD are: vasopressor use, inotropic therapy, mechanical ventilation duration and settings, P/F ratio, Aa gradient, need for continuous renal replacement therapy (CRRT) and, if applicable, duration of CRRT. Also, cardiac index will be measured before and after red cell transfusion with the help of a non-invasive Vigilance II. This measurement isn't part of standard care.

5.3.3 Blood samples

In addition to clinical characteristics and mitoPO2, we will measure Hb, haematocrit, arterial oxygen saturation (SaO2) and oxygen tension (PaO2), central venous oxygen saturation (ScvO2) if applicable and oxygen tension (PvO2) if applicable, and lactate in arterial and venous blood(if applicable) samples taken at the predefined moments before and after red cell transfusions. The collected blood will be immediately transported via the regular transport tubing system to the CKCL and CKHL for these analyses after being labelled with the unique subject number. Other measurements that will be done, in venous blood are: troponin, creatinine kinase, and creatinine. Urine will be collected for 24 hours on the day of transfusion itself and the day after, to calculate the glomerular filtration rate (GFR). Blood samples are already taken multiple times during a 24-h ICU stay, as part of a normal intensive care management. For this study troponin is not part of the daily routing of the ICU. Therefore, determination of troponin will be added. Appendix 2 gives an overview of measurements part of standard care, and which of those measurements are part of the study.

Information regarding ICU stay, hospital stay and mortality (ICU and hospital) will be available in and collected from the EPD. The follow up period will be three months. All data will be entered after validation in a study database for subsequent tabulation and statistical analysis. The data will be handled confidentially and anonymously. Patients re-admitted to the ICU and already participating in this study will only be followed according to the study protocol. Thus re-admitted patients will not be included for the second time.

5.3.4 Sample handling and Measurements:

Samples will be collected from

- central venous catheter (if applicable)
- arterial catheter

In order to make comparisons between different compartments of the body, blood samples (arterial, central venous) and cutaneous samples will be collected at predefined moments.

These moments during the pilot study are:
- Before transfusion (=T0)
- At the end of each transfusion (=T1)
- 30 minutes after T1 (=T2)
- 60 minutes after T1 (=T3)
- 180 minutes after T1 (=T4)
- 24 hours after T1 (=T5)

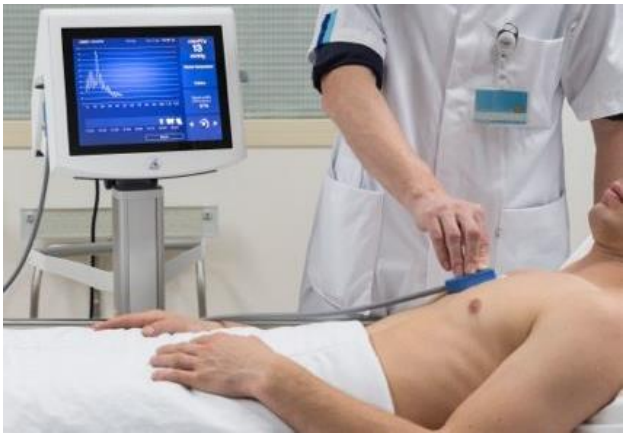

Figure3: Measurement MitoPO2 with the Comet

With the help of the pilot study, an overview of the measurements and parameters were made. With the help of this overview, decisions were made regarding the number of measurement moments (e.g. no need for measurement 15 minutes after red cell transfusion). Details about which parameters are determined at each time point during this pilot study are described in appendix 2. For the laboratorial measurements a total of 42ml of blood will be taken (10ml at T0 and T5, 4ml at T1 and 2, 6ml at T3 and 7.5ml at T4). As can be seen in appendix 2, most of the measurements are part of standard care which minimalizes the burden for the patient.

Analysis of red blood cell transfusion on the intensive care unit at LUMC showed 69% of transfusions occurred during daytime (between 8.00hr and 18hr). The remaining transfusions were evenly distributed in the evening and night time. Since transfusions outside the normal daytime are mostly because of urgent medical reasons, no measurements will be done in the evening or night.

Besides time of transfusion, analysis of previous ICU data showed that the mean duration elapsing between the decision of a non-emergency red cell transfusion and the start of the transfusion is 2-3 hours. Thus, maximum of 2 hours delay for start of transfusion during this study is achieved. However, at no time red cell transfusion will be delayed if the patient is clinically in need of a transfusion in order to have enough time to induce PpIX. To ensure this, the clinician responsible for the patient, and not the researcher, has the final decision in the timing of the red cell transfusion. The delay of transfusion will be recorded to gain an insight in delay of transfusion.

5.3.5 Other measurements

Also, cardiac output will be measured continuously by limited invasive methods such as the Vigileo/FloTrac.[27] This measurement is done with the help of an already placed arterial catheter. VO2, DO2 and oxygen extraction ratio will be calculated.
Sublingual side stream dark field imaging is the only non-invasive validated instrument to evaluate the microvascular perfusion in critical ill patients.[28,29] It will be used during this study to assess the microcirculation before and after red cell transfusion and compare this with the mitoPO2 measurements.

5.4 Study parameters

5.4.1 Main study outcomes

The primary endpoint is the change of mitoPO2 before and after red cell transfusion. This will be compared to change in traditional parameters used to measure oxygenation and oxygen balance (ScvO2, SaO2, PaO2, PvO2, CI, and lactate).

5.4.2 Secondary outcomes

Secondary study outcomes include:

- Value of mitoPO2 measurements in predicting (ischemic) organ damage. The following parameters for organ damage (that are routinely collected) will be assessed: lung (pO2/FiO2 ratio), heart (troponin, CK, an ECG, CI), renal (creatinine, urine production, glomerular filtration rate, RIFLE classification), brain (delirium, RASS) and SOFA score. More details about the timing of these parameters are given in appendix 2.
- Microcirculatory value of mitoPO2
- Duration of mechanical ventilation
- Need for renal replacements therapy
- Length of ICU stay
- Length of stay in hospital
- Hospital mortality
- ICU mortality
- 90-day mortality
- Assessment the safety of mitoPO2 measurements
    - Delay of transfusion because of mitoPO2 measurements
    - Adverse and serious adverse events of the mitoPO2 measurements

6. Statistical consideration

6.1 Power estimate

Since no prior mitoPO2 study had been performed on critically ill patients, no data was available to calculate the sample size needed for the prospective cohort study.
Therefore, we performed a pilot study with 20 subjects to assess feasibility of the mitoPO2 measurement and to calculate a standard deviation needed for the sample size calculation. The statistical significance level will be set at the 5% level. The sample size will be calculated to achieve a statistical power of 90%. We know from our pilot study performed on 20 critically ill patients that the mean mitoPO2 is 67.9 mmHg with a standard deviation of 13 mmHg. A difference in mitoPO2 of 5mmHg after red cell transfusion is deemed to be clinically relevant. Thus, the sample size calculation would subsequently be the following:
$Z_{\alpha/2} = 1.96$ (two tailed for $\mathrm{p} = 0.05$)
$Z_{1-\beta} = 1.28$ (for $\beta = 0.10$)
$\mu = 72.9\ mmHg$
$\mu_0 = 67.9\ mmHg$
$\sigma = 15\ mmHg$

$n = \left(\sigma \frac{z_{\alpha/2} + z_{1-\beta}}{\mu - \mu_0}\right)^2$ = 189. With an expected 10% drop out rate, the corrected sample size will be $189 * 1.1 = 209$ subjects.

6.2 Statistical methods to be employed

Absolute differences and 95% confidence intervals will be calculated. In addition to the univariate comparisons we will also calculate the differences for each of the parameters independent of the other parameters using multivariate models. In addition, we will perform similar analyses in subgroups of Hb and reason of admission (classified by APACHE IV) to assess whether the observed effect of transfusion on the parameters differs between the groups. We will also examine whether the decrease of the mitoPO2 precedes changes of other measures of oxygenation.

For the dichotomous outcomes logistic regression will be applied. For time-to event outcomes the univariable analysis will be performed using the Kaplan-Meier method, the multivariable analyses the Cox proportional hazard model. To determine the discriminative power of the model, the ROC curve with the c-statistic. P < 0.05 will be considered statistically significant for the primary outcome measure. The statistical analysis will be conducted using the SPSS (Statistical Package for the Social Sciences) statistical package, release 23.0 (SPSS Inc., Chicago, IL) and STATA.

7. Safety reporting

7.1 Section 10 WMO event

In accordance to section 10, subsection 1, of the WMO, the investigator will inform the subjects and the reviewing accredited METC if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited METC, except insofar as suspension would jeopardize the subjects' health. The investigator will take care that all subjects are kept informed.

7.2 Possible (serious) adverse events and suspected unexpected serious adverse reactions

The ALA patches are already used in and approved for the therapy of benign skin disorders with Photodynamic Therapy (PDT). Its use is safe and provides good treatment results with excellent cosmetic outcome. The 2x2cm large Alacare patches (8mg) were developed by Photonamic and are marketed for the treatment of actinic keratosis, a benign skin disorder, with photodynamic therapy. In dermatology, its use has been investigated.[10] In phototherapy, the treatment effect depends on the production of singlet oxygen by photoactivation of PpIX. Excitation of PpIX induces apoptosis in cells as a result of oxygen-radical formation. In general, this requires illumination with continuous light and a high cumulative light dose. In contrast, PpIX-TSLT uses short-pulsed excitation and a total light dosage, i.e., orders of magnitude less than used for photodynamic therapy.[10] A recent systematic review showed no significant (serious) adverse effects of ALA patches.[30]

Side effects of ALA described with photodynamic therapy are burning of the skin (<50%, resolved in 1-4 days), burning sensation of the skin (<92%), pruritus, hyperpigmentation (22-36%), edematous lesion (35%), application site erythema (92-99%), local pain (1-92%, <30% severe), application site irritation (72%), local haemorrhage(1-4%), and headache (<10%). Most of these reactions resolved within 2 weeks. There are no reported (serious) adverse events.[16,22,23] Since the light load at the cutaneous-PpIX-TSLT is much lower than in phototherapy, no adverse events are expected. While PpIX-TSLT is likely to be safe, the effects of phototoxicity after PpIX induction should always be considered a potential risk requiring a risk and safety assessment in any intended application.[10]

7.3 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the investigational product. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

7.4 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);

- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- Any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have, based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

The investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events. Patients on the ICU are critically ill and therefore more prone to SAEs. ICU mortality in the Netherlands is approximately 8.4%. Thirty-five percent of the patients are in need for vasopressor therapy, and 46% of the patients are mechanically ventilated. Furthermore, the National Intensive Care Evaluation Report of 2015 reports that the mean ICU admittance duration is 3 days with a mean in hospital admittance duration of 13 days. Around 80% of the total ICU patients have a low APACHE IV score, and therefore a mortality chance of less than 30%.[31] Thus, SAEs in relation to underlying disease and expected SAEs during the ICU course will not be reported.

The coordinating investigator will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

7.5 Suspected unexpected serious adverse reactions (SUSARs)

Adverse reactions are all untoward and unintended responses to an investigational product (in this case the ALA patch as part of the mitoPO2 measurement) related to any dose administered. No SUSARs of the ALA patch are reported. Therefore, no SUSARs are expected during this study.

Unexpected adverse reactions are SUSARs if the following three conditions are met:

1. the event must be serious (see chapter 7.4);
2. there must be a certain degree of probability that the event is a harmful and an undesirable reaction to the ALA patch, regardless of the administered dose;
3. the adverse reaction must be unexpected, that is to say, the nature and severity of the adverse reaction are not in agreement with the product information as recorded in:
   - Summary of Product Characteristics (SPC) for the COMET device;
   - Investigator's Brochure for the ALA patch.

The coordinating investigator of LUMC will report expedited the following SUSARs through the web portal *ToetsingOnline* to the METC:

- SUSARs that have arisen in the clinical study that was assessed by the METC;

- SUSARs that have arisen in other clinical studies of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical study that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line-listing provides an overview of all SUSARs from the ALA patch, accompanied by a brief report highlighting the main points of concern.
The expedited reporting of SUSARs through the web portal *ToetsingOnline* is sufficient as notification to the competent authority.

The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

The coordinating investigator of LUMC is responsible for documentation of the SUSAR in the report and ToetsingOnline.nl. The principal investigators in the participating centres will inform the coordinating investigator in <24 hours after the occurrence of the SUSAR. In the case of occurrence of 2 or more SUSARs in 6 months' time, the study team will discuss possible termination of the study.

7.6 Annual safety report

In addition to the expedited reporting of SUSARs, the coordinating investigator will submit, once a year throughout the clinical study, a safety report to the accredited METC. This will be combined with the annual progress report.
This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system.
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the ALA patch.

7.7 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported untill end of study within the Netherlands, as defined in the protocol

7.8 Data Safety Monitoring Board

This is a moderate risk study in which the side effect of ALA are well described. The side effects are based on studies with photodynamic therapy. Since the total lightload in photodynamic therapy is significantly higher and longer than during the measurements in this study, no SAEs and SUSARs are

expected.[10,32] Indeed, studies using the ALA plaster for mitoPO2 measurement in Rotterdam reported no serious adverse events and no SUSARs.[21,33,36] These studies were performed on healthy volunteers,[21] surgical patients[33] and patients with chronic anaemia.[40] However, these studies reported local side effects of the ALA plaster consisting of erythema, pruritus and transient hyperpigmentation. All these effects were temporary and disappeared mostly within 1-2 weeks' time. No local side effects or other adverse events were seen in our pilot study. To ensure the safety of our study participants, monitoring will take place during the study. Furthermore, the in- and exclusion criteria are optimised to minimize the risk for participants. Since safety is part of our secondary outcome, we will have attention for AEs, SAEs and SUSARs. With an independent expert on data safety monitoring board confirming the needlessness of a DSMB for the INOX ICU-2 study, no DSMB was initiated.

8. Ethical considerations

8.1 Regulation Statement

The study will be conducted according to the principles of the Declaration of Helsinki (accepted by the WMA General Assembly, 9 October 2004, Tokyo) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

8.2 Recruitment and Consent

The protocol of this study will be submitted to the Medical Ethics Committee of the Leiden University Medical Centre. The study will not commence before formal approval has been granted.

Since research has shown that approximately 10% of critically ill patients can make autonomous decision regarding research participation, most decisions will be made by their substitute decision makers, who are already overwhelmed by the medical information of the patient itself. Furthermore, studies have shown that obtaining contact with their substitute decision makers can be difficult in 7%, no decision maker exist in 6% of the cases. In addition, approximately half of the opportunities to recruit patients are missed because of workload and narrow time windows for inclusion. Difficulties in recruitment of eligible patient can lead to delayed identification of (in)effective treatment/diagnostics and limitation of generalizability. Alternative consent models are therefore needed to improve recruitment and minimize withdrawal.[24,25] Therefore, we will firstask all patients admitted to the ICU, or their legal representatives if the patient is not able to give his/her consent, expected to stay at the ICU for longer than 24 hours for participation in our study. Patients admitted to the cardiothoracic department which will be admitted to the ICU post-operatively, will be asked for an informed consent on the day before the cardiothoracic operation. An information leaflet will be given and informed consent will be sought. Patients or their legal representatives will be told that a person can only participate in the study if he/she meets the inclusion criteria.

However, if the patient or legal representative could not have been reached in time for written informed consent, while the patient met the eligibility criteria, patient will be included with deferred consent procedure. Informed consent will be sought within 48 hours after inclusion of the patient.Deferred consent is therefore only used in situations where it is not possible to ask informed consent, including the following: lack of decision-making capacity of the patient due to illness severity, delirium, use of sedatives, analgesics and life-sustaining treatments; inability to contact the legal representative; overwhelming nature of medical information for the legal representative therefore influencing the capability of informed decision-making.[24,34] aAt the moment the decision is made to administer a red cell transfusion, the self-adhesive ALA-patch will be placed, if the patient meet the eligibility criteria, and data collection for the study started.Due to logistics, the normal time elapsing between the decision of a non-emergency red cell transfusion and the actual administration is 2 to 3 hours. Therefore, the maximal delay of a non-emergency transfusion will be 1 to 2 hours. To ensure improper delay of red cell transfusion due to the study, the clinician responsible for the patient, and not the researcher, has the final decision in the timing of the red cell transfusion.

If written informed consent was given, but retracted by legal representative or patient after participation, the patient will be excluded and data will no longer be used. If patient or legal representative denies participation during the study, data collected until that time point will be used in the analysis. No further data will be collected.

8.3 Objection by minors or incapacitated subjects

Section 4, subsection 2, of the WMO stipulates that a legally incompetent adult cannot be forced to undergo a treatment or behave in a particular manner in the context of non-therapeutic research against his or her will. Subjects younger than 18 years are already excluded from the study. If there are adults, that are incapable of giving informed consent, a legal representative will be asked to give informed consent according to the GCP guideline. When the subject is able to give informed consent after being included in the study by consent of the legal representative and denies any further participation, the subject will be excluded from the study without any consequences. The data collected up to that point will be used for analysis. No additional data will be collected.

8.4 Benefits and risk assessment, group relatedness

The proposed study aims to optimize transfusion strategies in critically ill patients with anaemia. The measurements necessary to assess the defined study endpoints are not expected to negatively influence the result of treatment.

8.5 Compensation for injury

The Leiden University Medical Centre has a liability insurance which is in accordance with article 7, subsection 6 of the WMO.

The LUMC (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23th June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.

1. € 650.000, -- (six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
2. € 5.000.000, -- (five million Euro) for death or injury for all subjects who participate in the Research;
3. € 7.500.000, -- (seven million and five hundred thousand Euro) for the total damage incurred by the organization for all damage disclosed by scientific research for the Sponsor as ‘verrichter’ in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

9. Administrative Aspects and Publication

9.1 Handling and storage of data and documents

All patients will be addressed to with a random patient identification code, which will be generated by CASTOR EDC, the electronic case report form. The codebook will be stored digitally, outside the investigator site file folder, with restricted access. Only the research team, monitor, accredited METC and IGJ will have access to this folder. The code will be safeguarded by the coordinating investigator. The codebook will be encrypted with a password. All data will be stored for further publication. All handling of personal data will comply with the EU General Data Protection Regulation (GDPR) and the Dutch Act on Implementation of the General Data Protection Regulation (in Dutch: Uitvoeringswet AVG). Source data on the COMET device will be anonymous, it only contains date and time of measurement, it is not possible to enter patient name, personal number or date of birth. The COMET can only retrieve the data of the current/most recent measurement series until the user selects a new series or measurement spot. To store COMET data, it will be downloaded via an USB memory onto a computer memory conforming to the institution's privacy guidelines and the USB memory will be erased. The COMET data will be shared anonymously with the manufacturer.

9.2 Monitoring and Quality assurance

The risks associated with this study are moderate for the subjects (more information can be found in chapter 10 and in annex 4). Therefore, only on-site monitoring is needed, which will happen in accordance of the NFU guideline of monitoring. Being labelled as a moderate risk study, the monitoring will be moderate to ensure the integrity and safety of the study participants. The monitorpool of Leiden University Medical Center, which are qualified for monitoring, will monitor this study. The monitorpoolis an independent group of data managers or research nurses, who aren't involved in the study in any way other than monitoring. They will report to each principal investigator per site and to the coordinating investigator. The head of department of each site will be notified by the monitor, when the monitor notices frequent or substantial omissions.

9.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion. All substantial amendments will be notified to the METC and to the competent authority. Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

9.4 Annual progress report

The investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects

included and numbers of subjects that have completed the trial, unexpected problems and amendments.

9.5 End of study report

The investigator will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the 90th day after the last patients' admittance to the ICU. In case the study is ended prematurely, the investigator will notify the accredited METC within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

9.6 Public disclosure and publication policy

The study protocol and analysis plan will be published before start of the study on clinicaltrials.gov. The results of the study will find their way into (inter–) national scientific journals and guidelines.

10. Structured risk analysis

In the following analysis we distinguish potential issues of concern related to the mitoPO$_2$ measurements

a. Level of knowledge about mechanism of action

The mechanism of action of mitochondrial oxygen tension measurements is well investigated since 2006 and published in several articles.[10,17-19,35]

Compared with other techniques to measure oxygen at the tissue level, oxygen-dependent quenching of delayed fluorescence of PpIX has some distinct advantages. It is quantitative, and, unlike oxygen electrodes, once calibrated it does not need recalibration at the time of usage. As opposed to oxygen saturation measurements based on near-infrared spectroscopy, the measurement site within the tissue is well defined. Therefore, the signal is not sensitive to changes in vessel density, like capillary recruitment. Furthermore, the technique relies on lifetime measurements instead of intensity measurements and therefore is highly insensitive to changes in tissue optical properties occurring, e.g., in case of venous congestion.[10]

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

Topical application of ALA is registered for use of photodynamic therapy of skin lesions. Its use for measuring mitoPO$_2$ in healthy volunteers has been proven to be safe in the finished study with protocol number NL37911.078.11 in which the PpIX-TSLT technique was tested on 30 healthy volunteers. All healthy volunteers experienced the skin preparation and measurements as non-problematic. Due to either the skin preparation or the ALA patch, 45% of the volunteers suffered from mild pruritus and/or erythema on the actual measurement day; these minor complaints were no longer present the day after the measurements. Only two volunteers had transient hyperpigmentation of the skin after the measurements, possibly due to premature exposure of the primed area to sunlight (against our advice). The hyperpigmentation was temporary and disappeared within one month. None of the volunteers sustained long-term skin damage, as established one month after the experiments.[21]

It was also used in finished study with protocol number NL56686.091.16 in May and June 2016 at UMC St. Radboud in Nijmegen. This study induced endotoxaemia in human volunteers. Among other measurements, the COMET measurement system and Alacare were used to measure oxygen availability and consumption. ALA was applied on the evening before the endotoxaemia day. The study again confirmed the safety profile of the COMET's measurement also during endotoxaemia. The measurements of the COMET identified change of mitochondrial function in vivo during human endotoxemia.[36]

Other protocols use the same measuring technique: The running studies with Protocol nr: NL51187.078.14 (Photodynamic therapy study) and Protocol nr: NL51937.078.15 (Non-invasive monitoring of mitochondrial oxygen consumption and oxygenation (COMET): observational clinical

study) and NL55664.078.15 (MOTIFATE pilot, a study of blood transfusion and fluid administration in chronic anaemia).

c. Can the primary or secondary mechanism be induced in animals and/or in ex-vivo human cell material?

Mitochondrial oxygen tension research has been performed in cells and animals prior to this research proposal.[13,14,32,37,38] PpIX-TSLT is one of the central techniques used in translational research in the Laboratory of Experimental Anaesthesiology of Erasmus Medical Center.

d. Selectivity of the mechanism to target tissue in animals and/or human beings

Not applicable

e. Analysis of potential effect

The PpIX-TSLT technique as well as the COMET have been investigated and have been proven safe, as demonstrated by studies on volunteers and patients. The possible effect of phototoxity after PpIX induction is a potential risk, but because the PpIX-TSLT uses short-pulsed excitation and total light dosage is much less than used in photodynamic therapy, this risk is considered to be very low.[17,39] Therefore we expect no effects of the measurement for the patients (aside from reported temporary mild discomfort like erythema and pruritus associated with the topical application of ALA).

f. Pharmacokinetic considerations

Not applicable

g. Study population

Critically ill patients on the ICU are vulnerable patients. According to GCP guidelines, a non-therapeutical study can only take place if it has a likelihood of benefit for group represented by the subject. With the results of this study a potential phase 2 study can be performed to tailor the transfusion strategies in critical ill patients to reduce both over- and under- transfusion. Besides the benefit for critically ill patients, this study entails moderate risk and burden for the subjects since it is a non-invasive measurement with no known serious adverse reactions.

Some patients will have an altered consciousness which makes informed consent impossible in these patients. Therefore, we will seek informed consent from the legally authorised representative according to the GCP guidelines.

To minimalize delay of transfusion, the ALA patch will be placed after the indication for red cell transfusion is made and informed consent is present. In case of situations outlined in chapter 8.2, informed consent will be sought as soon as possible after inclusion, but no longer than 48 hours after inclusion. If no written informed consent is achieved, subjects will be excluded from the study. The clinician responsible for the patient, not the researcher, makes the final decision in the timing of the red cell transfusion.

h. Interaction with other products

None expected, see Alacare product information.[16]

i. Predictability of effect

No effect of the measurements is expected. Minor temporary skin lesions can occur, as seen in the healthy volunteers. With the COMET we expect less effect, due to progressive technique since the study in healthy volunteers, so the light intensity and total light dosage will be even less.

j. Can effects be managed?

No effects of the mitoPO_2 measurements are expected, but when there is any indication of a skin lesion (e.g. unexpected pain during the measurements), the measurements can and will be stopped.

11. List of abbreviations

AE	Adverse Event
ALA-patch	Cutaneous patch with 5-aminolevulinic acid
AR	Adverse Reaction
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CKCL	Central clinical chemical laboratory
CKHL	Central clinical haematological laboratory
CI	Cardiac index
DO2	Oxygen delivery
DSMB	Data Safety Monitoring Board
EPD	Electronic patient dossier
GCP	Good Clinical Practice
GFR	Glomerular filtration rate
IC	Informed Consent
ICU	Intensive Care Unit
METC	Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)
MitoPO2	Mitochondrial oxygen tension
NFU	Nederlandse Federatie Universitair Medisch Centra
PaO2	Arterial oxygen tension
PDT	Photodynamic therapy
PpIX	Protoporphyine IX
PvO2	Venous oxygen tension
RIFLE	Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease classification
(S)AE	(Serious) Adverse Event
ScvO2	Central venous oxygen saturation
SaO2	Arterial saturation
Sponsor	The sponsor is the party that commissions the organization or performance of the research, for example a pharmaceutical company, academic hospital, scientific organization or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidizing party.
TSLT	Triplet State Lifetime Technique (protoporphyrin IX-triplet state lifetime technique)
VO2	Oxygen consumption
WBP	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

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13. Appendix 1

Background on the measurement

Photonics Healthcare B.V. (PH) has developed an innovative non-invasive bedside monitoring system to measure Cellular Oxygen METabolism (the COMET). This device, for the first time, provides insight in the adequacy of oxygen supply and the actual oxygen demand. Available technologies only measure parameters in the blood, where oxygen is transported, but not where it is actually needed. The COMET measures availability and utilization of oxygen where oxygen is utilized: in the tissue cells.

The COMET measurement system consists of three parts:
1. The bedside COMET monitor: has a multi-touch screen that directly, in real time, displays the mitoPO2, the functional parameter of mitochondrial oxygen availability in mmHg. Additionally, signal quality and sensor temperature indication at the time of the most recent oxygen measurement are shown. The user can set the measurement interval and follow the reaction of cellular oxygen to various interventions, or perform a spot-check, a single measurement on a specific part of the skin of the situation at a particular time. A measurement series in a situation where pressure temporarily stops local blood flow can measure oxygen utilization. The device is a standalone unit that can be mounted on a moveable cart or used on a table.
2. The COMET Skin Sensor: is optically and electrically connected with the COMET measurement device. The optical fibers transmit optical signals for tissue excitation and fluorescence detection. The Skin Sensor creates a diverging excitation pulse allowing a superficial optical measurement of the oxygen in the mitochondria of the cells in the epidermis in a circular area of about 5 mm2. Fluorescence light from the tissue is collected and transmitted via the detection fiber towards the detector system. The sensor also detects ambient light and the temperature inside the sensor.
3. ALA patch and sensor fixation: The dermatologic agent 5-aminolevulinic acid (ALA) is applied to the patient skin. The sensor is fixated to the site with the help of a patch on which the skin sensor is placed. Simple ALA patches were developed by Photonamic and are marketed for photodynamic therapy, ALA crèmes are available from Galderma, photocure, DUSA and Biofrontera. This set up allows the physician to place the ALA-patch anywhere on the patient's skin and, after four hours, place the sensor to locally determine the mitoPO2 level in the cells of the epidermis, which is then displayed on the monitor. Measurements can be performed every second for several minutes, or every few minutes for a period of 24 hours on the same spot of skin. After that, another location for the patch should be chosen.

The measurement specifications:
Photonics Healthcare's COMET monitor measures absolute oxygen availability precisely and exactly where it is needed: in the mitochondria of the active cells. This provides doctors with quantitative insight in the availability and consumption of oxygen in their patients' cells and hence the need for a blood transfusion. To determine the oxygen tension in the mitochondria, the COMET measures the

lifetime of the triplet state of protoporphyrin IX (PpIX), a precursor of haeme and hence haemoglobin. The discovery that PpIX exhibits a 'glow-in-the-dark' effect with an oxygen-dependent life time was published in a Nature journal; patents are awarded in Europe and the US and exclusively licensed by PH. The COMET probes the tissue with very short pulses of green light generated by a diode-pumped solid state laser. This light is brought to the site via an optical fibre. The COMET then observes the decay of the intensity of delayed fluorescence from the red spectrum of light collected from the tissue in the first milliseconds after the flash. Under normal conditions, PpIX is present in very low concentrations due to the presence of a negative feedback loop in which a product of PpIX, haeme, acts to inhibit the production of aminolevulinic acid (ALA), a substrate for PpIX. This can be overcome by the exogenous administration of ALA, which on its turn leads to higher concentrations of protoporphyrin IX in the mitochondria.[1] ALA patches are already used in and approved for the therapy of benign skin disorders with Photodynamic Therapy (PDT). Its use is safe and provides good treatment results with excellent cosmetic outcome. The 2x2cm large Alacare patches (8mg) were developed by Photonamic and are marketed for the treatment of actinic keratosis, a benign skin disorder, with photodynamic therapy.

The key specifications of the COMET sensor system are:
-Detection range: 0 – 200 mmHg (arterial oxygen tension does not exceed 100 mmHg in normal physiologic situations).
-Tolerance of Monitor: +/- 15%, at least +/- 3 mmHg, for signal quality >75%, which is similar or better than many clinical measurement systems such as cardiac output (+/- 30%), tissue oxygen (+/- 30%) or haemoglobin (+/-20%)[2,3]. Repeatability and stability of the measurement is high and absolute accuracy will improve as more information about the new measurement becomes known but already is higher than for some current parameters used in the clinic (for example cardiac output).
-Time to result: 1 second (for a prepared measurement location, i.e. placement of ALA-patch 4 hours previously to measurement).
-Simple handling (one hour of training is sufficient to train any doctoral student, nurse or doctor)
-All-in-one system, no need for other equipment or control mechanisms.

Current stage of development:
The COMET measurement system has been developed based upon applicable regulations, standards (Medical device directive 93/42EEC, IEC 60601 rev 3, ISO 13485) and has been tested in preclinical tests and volunteer studies. The key milestones are described below.

The crucial step in the invention is the exploitation of the oxygen dependent optical properties of protoporphyrin IX. Because this substance is made in the mitochondria and can be measured on the skin, the problem of signal location that has made all earlier attempts to directly measure cellular metabolism unsuccessful is solved. First publication of the feasibility of the measurements was in 2006 in Nature Methods.[4]

Subsequently the methods was tested in vivo on various organs,[5-7] validated against existing technologies[8,9] and showed the measurement could be used to measure oxygen consumption i.e., cellular energy turnover in a first human volunteer.[10,11] Further studies demonstrated that cellular oxygen availability and consumption can safely be measured using ALA patches and COMET prototypes in a study in 30 human volunteers at Erasmus MC in Rotterdam.[12] it was shown that information from the skin is representative for other parts of the body.[13] From September 2014 until April 2015, Van Diemen et al. measured the increase of cellular oxygen consumption with the COMET prototype in skin of 28 human volunteers after 4 weeks of daily statin use (Simvastatin, a cholesterol lowering drug suspected of modifying mitochondrial function) and its partial reversal by additional intake of ubiquinol (Q10), which contributes to mitochondrial function.[13] Next to the COMET the study also measured phosphocreatine recovery time after muscle exercise in 7-Tesla-31P-MR spectroscopy and mitochondrial membrane potential in white blood cells. The practically perfect correlation of the results in all groups is a strong validation of PH's measurement against a very expensive and cumbersome technique.

PH developed the COMET monitor, the first clinical monitor for cellular oxygen and conducted preclinical research with the help of partners, a Eurostars grant and investors.[14,15]

In patients receiving ALA assisted photodynamic therapy and during neurosurgery oxygen levels were measured with the COMET. These demonstrate that the COMET can be used in patients and measurements are stable. The study in this project will take the first step towards the use of this technology for blood transfusion in critically ill patients.

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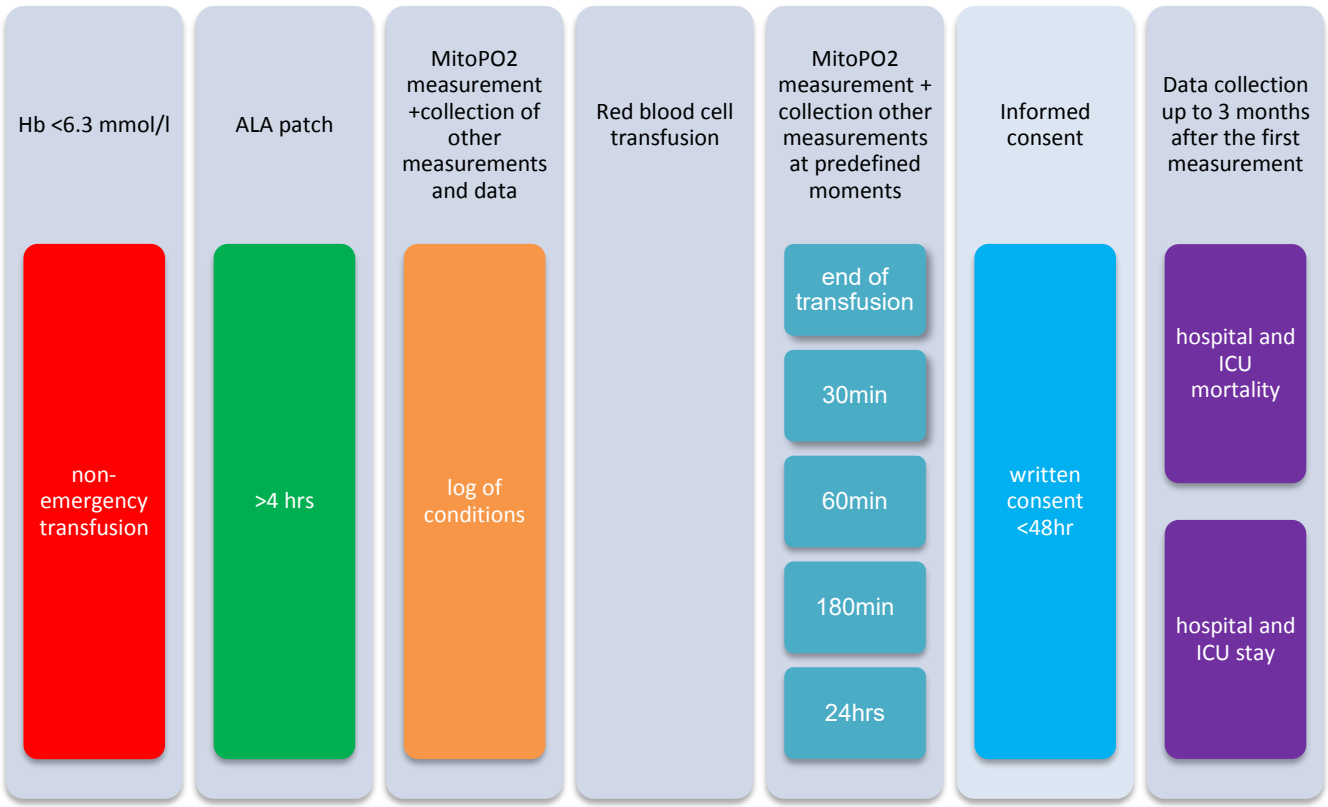
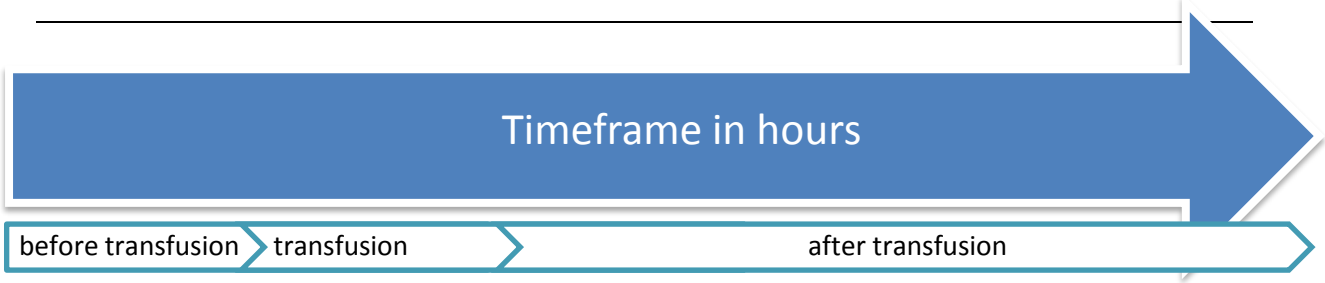
15 E!7294 COMET was awarded in 2012, Photonics Healthcare raised funds from investors including High-Tech Gr nderfonds in 2013.

14. Appendix 2: Overview of measurements



Standard measurements	•**Daily** •creatinin, Hb, Ht, lactate, SaO2, ScvO2, PvO2, PvCO2, PaO2, PaCO2 •ECG •APACHE II and IV (at admission), SOFA, RIFLE •**Continu** •SpO2, HF, BP, CVD, UP, ICDSC, RASS, GCS •inotropic therapy, vasopressor use •fraction of inspired oxygen, mechanical ventilation duration and setting, renal replacement duration and settings
Extra determinations	•troponin •CK
Extra measurements	•mitoPO2 at T0-T5 •ScvO2, SaO2, PaO2, PaCO2, PvCO2, PvO2 and lactate at T1-4 •Hb measurement at T3 •Cardiac index measurement with Vigileo II at T0 and T5 •Microcirculation with sublingual SDF imaging at T0 and T5



ALA patch ≥4 hrs (=T0)

End of (each) transfusion (=T1) **T2** **T3** **T4**

24hrs after T1 (=T5)

MitoPO2

MitoPO2 measurements on T1-5

Demographic data:
-age and gender
-height+ weight
-allergies
-comorbidity
-medication use
-smoking
-reason ICU admission
-NYHA class
-APACHE II and IV
-pulmonary function

Clinical measures T0:
-HF
-BP
-CVP
-SpO2
-GCS
-UP+FB
-ECG
-RIFLE
-SOFA
-RASS
-ICDSC score
-CI

-Sublingual SDF

Collection measurements on T1-4:
-HF
-BP
-CVP
-SpO2
-GCS
-UP+FB
-ECG
-RASS

Clinical measures T5:
-HF
-BP
-CVP
-SpO2
-GCS
-UP+FB
-ECG
-RIFLE
-SOFA
-RASS
-ICDSC score
-CI
-Sublingual SDF

Blood sample T0:
-creatinine
-troponin
-CK
-Hb
-Ht
-lactate
-SaO2
-ScvO2
-PaO2 + PvO2
-PaCO2 + PvCO2
Urine for 24 hours

Blood sample on T1-T4:
-lactate
-SaO2
-ScvO2
-PaO2
-PvO2

On T3:
- Hb

On T4:
- Troponin and CK

Blood sample T5:
-creatinine
-troponin
-CK
-Hb
-Ht
-lactate
-SaO2
-ScvO2
-PaO2
-PvO2
Urine for 24 hours

Collection of other clinical data

- (duration of)mechanical ventilation, FiO2, P/F ratio, Aa gradient
- inotropical therapy
- vasopressor use
- renal replacement therapy

Abbreviations: *BP*=blood pressure; *CK*= creatinine kinase; *CI*= cardiac index; *CVP*=central venous pressure; *ECG*= electrocardiogram; *FB*= fluid balance; *FiO2*= fraction inspired oxygen; *GCS*= Glasgow coma scale; *Hb*= haemoglobin; *HF*=heart frequency; *Ht*= haematocrit; ICDSC= intensive care delirium screening checklist; *ICU*=intensive care unit; *NYHA*= New York Heart Association class; *PaO2*= arterial oxygen tension; *PvO2*= venous oxygen tension; *RASS*=Richmond agitation- sedation scale; *RIFLE*= Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease classification ; *SaO2*= arterial saturation; *ScvO2*= central venous oxygen saturation; *SDF*= side stream dark field imaging; *SOFA*= sequential organ failure assessment; *SpO2*=peripheral oxygen saturation; *UP*= urine production.

15. Appendix 3: Skin Pad

1. Description

The Skin Pad is a non-sterile, single use device to forcelessly hold a sensor in optical access on intact skin for no more than 24 hours.

The fixation of a sensor with the Skin Pad is designed to provide a steady and reliable measurement and allow repeated or continuous measurements over longer time periods that do not require the user to hold the sensor of fix it with tape that might cause changes to the signal.

The Skin Pad on the skin with a sensor attached is shown in Figure 1.

The Skin Pad consists of three layers and a release liner on each side to protect the adhesive surfaces before use.

The central layer is a thin poleurethane film, double coated with a silicone gel adhesive and anacrylic adhesive on the opposite side (Scapa Soft Pro 6051). The silicon adhesive layer covering the entire surface area of the “skin side” of the Skin Pad is covered with a yellow LDPE release liner (Figure 2). Once this release liner is removed the silicon adhesive makes it possible to attach the sensor on the desired location on intact skin and reposition it several times.The adhesive power of the silicon adhesive is designed for sensitive skin and is called “skinfriendly”.

The “sensor side” of the Skin Pad is covered with a white PE foam (Scapa 9868/824) except for a central part with the dimensions of the sensor where the acrylic adhesive is exposed and where the sensor should be attached. The exposed acrylic adhesive is covered with a whiterelease liner with a lip for easy removal before the skin sensor is attached.

Small notches on the side of the Skin Pad can assist in orientation. For a sensor with an active measurement area 14 mm from the end of the sensor, these notches indicate the location of the measurement area in the midline of a sensor attached inside the clear adhesive area surrounded by the white foam. The Skin Pad should then be attached to the skin so that the mid- line of the Skin Pad and the line connecting the notches intersect at the center of the desiredmeasurement location.

Between the film and the foam, not accessible to the user or patient, is a metalized white poly-propylene film fixed to the film with a medical-grade rubber adhesive (Bioflex Rx848P). This layer will largely block ambient light to reduce the noise for the measurements.

Figure 1: Skin Pad on the arm fixating a sensor next to a measurement device

Figure 2: Yellow release liner of Skin Pad

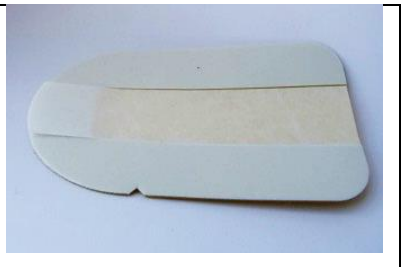


Figure 3: Top view of sensor with white release liner

1.1 Biocompatibility

All materials in the Skin Pad are biocompatible and tested according ISO 10993-1:2009 Biological evaluation of medical devices. Part 1: Evaluation and testing in the risk management process. All materials of the Skin Pad are considered non-cytotoxic (grade 0, ISO 10993-5) and negligible irritant (irritation score 0, ISO 10993-10).

1.2 Non-Sterile

The Skin Pad is a non-sterile product to be used on intact skin.

1.3 Usage

The Skin Pad is a single use product.

1.4 Material datasheets

Double coated layer: SP_datasheet
6051.pdf Foam layer: SP_datasheet 9868-
824.pdf
Aluminum layer: SP_ datasheet RX848P TDS Format.pdf

2. Intended use

The Skin Pad is a non-sterile, single use device to forcelessly hold a sensor in optical contact to intact skin for no more than 24 hours.

3. Device Class

The Skin Pad is a Class I device according to MDD 93/42 Rule 1 and a non-invasive device according to the medical devices guidance 'MEDDEV 2.4/1 Rev.9 Juni 2010'. CE mark is pending.

4. History of Document and Responsibility

Versio	Date	Responsible	Reason
1	17-12-2013	Rinse Ubbink	Initial creation
2	7 Oct 2015	Rinse Ubbink	Updated according to new design
3	7-Oct-2015	Michael Münker	Updated description, generic

16. Appendix 4: Monitor plan according to NFU-guidelines

	Moderate risk but intensive monitoring
Monitor frequency	3 visits per year per centre
Patientflow[1]	Inclusion speed and percentage of patients fallen out (e.g. withdrawn, loss to follow up)
Trial Master File / Investigator File	Presence and integrity of investigation file
Informed consent	25%
In-/exclusion criteria	25% of all subjects of each centre[2]
Source Data Verification	25% (Based at predefined list of variables, including primary endpoint, which are in clear relation to safety and validity of the study)
SAE[3]	100% of the total subjects for possible missed SAE. It includes verification of procedures as well.[3]
Investigated product	Control of instructions given to subjects. Also, control of delivery, distribution, storage, return, expiration date and timely order of the ALA patch
Study procedure	Control of presence of instructions needed for study procedure. If needed, facilities and equipment will be checked as well. An initiation visit will be performed in each centre before start of the study in that centre.
Laboratory	Control of certification of laboratory (GLP certification)
Biological monsters	Control of assemblage, labelling and storage

1 Monitoring of patient flow regardless of the risk classification since inclusion speed going to slow can threaten completion of investigation.

2 If subjects are included wrongly, all files of the centre involved with that will be reviewed and controlled.

3 If the reporting and / or appropriate reporting of serious adverse events (SAE) is incomplete or incorrect, all files of that particular centre need to be checked, regardless of the degree of intensity of monitoring.