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Study protocol and pilot results of an observational cohort study evaluating effect of red blood cell transfusion on oxygenation and mitochondrial oxygen tension in critically ill patients with anaemia: the INsufficient Oxygenation in the Intensive Care Unit (INOX ICU-2) study

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ABSTRACT

Introduction The recently developed protoporphyrin IX-triple state lifetime technique measures mitochondrial oxygen tension (mitoPO2) in vivo at the bedside. MitoPO2 might be an early indicator of oxygen disbalance in cells of critically ill patients and therefore may support clinical decisions regarding red blood cell (RBC) transfusion. We aim to investigate the effect of RBC transfusion and the associated changes in haemoglobin concentration on mitoPO2 and other physiological measures of tissue oxygenation and oxygen balance in critically ill patients with anaemia. We present the protocol and pilot results for this study.

Methods and analysis We perform a prospective multicentre observational study in three mixed intensive care units in the Netherlands with critically ill patients with anaemia in whom an RBC transfusion is planned. The skin of the anterior chest wall of the patients is primed with a 5-aminolevulinic acid patch for 4 hours for induction of mitochondrial protoporphyrin-IX to enable measurements of mitoPO2, which is done with the COMET monitoring device. At multiple predefined moments, before and after RBC transfusion, we assess mitoPO2 and other physiological parameters of oxygen balance and tissue oxygenation. Descriptive statistics will be used to describe the data. A linear mixed-effect model will be used to study the association between RBC transfusion and mitoPO2 and other traditional parameters of oxygenation, oxygen delivery and oxygen balance. Missing data will be imputed using multiple imputation methods.

Ethics and dissemination The institutional ethics committee of each participating centre approved the study (reference P16.303), which will be conducted according to the 1964 Helsinki declaration and its later amendments. The results will be submitted for publication in peer-reviewed journals and presented at scientific conferences.

Strengths and limitations of this study

- The study includes consecutive, critically ill patients with severe anaemia receiving red cell transfusions that ensure generalisability to patients in whom oxygenation monitoring at the cellular level is most relevant.
- In addition to all the macrocirculatory measurements for tissue oxygenation in critically ill patients, the study will examine bedside oxygenation at the cellular level with the mitochondrial oxygen tension (mitoPO2).
- Measurements are done at various timepoints before and after red blood cell transfusion that will generate data on whether changes in cellular oxygenation parameters—as assessed with mitoPO2—precede changes in macrocirculatory measurements for tissue oxygenation.
- The diagnostic value of mitoPO2 cannot be validated against a gold standard, because this is the first test to measure cellular oxygenation in patients.
- Patients in need of an immediate transfusion cannot be included, because the measurement can only be performed 4 hours after application of the patch.

Trial registration number NCT03092297.

INTRODUCTION

Adequate tissue oxygen delivery is one of the cornerstones of therapy in critical care medicine. However, direct measurements of tissue oxygenation that would inform the clinical assessment of critically ill patients at the bedside are lacking. Currently, we...
monitor tissue oxygenation by measuring global oxygen delivery, and one of the most important determinants of global oxygen delivery is haemoglobin (Hb). In clinical practice, Hb is used as a surrogate marker for the need for red blood cell (RBC) transfusion to treat an oxygen deficit, but personalised Hb concentrations at which oxygen deficits exist are still uncertain. Furthermore, transfusion does not necessarily improve tissue oxygenation, indicating that other oxygenation parameters are needed to determine the need for RBC transfusion.

A series of new techniques have been developed to quantify oxygen content in the microcirculation and within the cell. The protoporphyrin IX-triple state lifetime technique (PpIX-TSLT) measures mitochondrial oxygen tension (mitoPO2) through the oxygen-dependent optical properties of protoporphyrin IX (PpIX). Thus far, the potential of PpIX-TSLT as an early indicator of oxygen deficit in the cell has been shown in animal models, where after the feasibility has been studied in healthy volunteers, patients undergoing gastroscopy and in a neurosurgical patient. Its ability to measure mitoPO2 has also been validated in vivo in various organs and tissues. However, whether PpIX-TSLT can be used to measure cellular oxygenation in critically ill patients has not been assessed yet.

We aim to investigate the effect of RBC transfusion on mitoPO2 and the association between mitoPO2 and other physiological parameters of oxygen balance and tissue oxygenation in anaemic critically ill patients. In this report, we describe the study protocol of this observational study, the study programme in terms of pilot and main study and how preliminary results of the pilot study resulted in adjustment of the main study design.

METHODS AND ANALYSIS
This manuscript was written in accordance with the Standard Protocol Items Recommendations for Interventional Trials.

Study design and setting
The INsufficientOXygenation in ICU patients-2 is a multicentre observational cohort study running in three academic hospital intensive care unit (ICU) departments in the Netherlands: Leiden University Medical Center, Amsterdam University Medical Center and Erasmus University Medical Center Rotterdam. The participating ICUs are all ICUs with a mixed medical and surgical patient population. Enrolment for this study started in May 2017 for the pilot study solely in Leiden University Medical Center, after which enrolment for the main study started in March 2018. The pilot study was designed to investigate whether crucial components of the main study—such as the measurement of mitoPO2 in critically ill patients with anaemia and inclusion of eligible patients—were feasible and aimed to predict an appropriate sample size for the full-scale project and to improve the study design. The study was registered in ClinicalTrials.gov prior to study enrolment. Informed consent is asked by a study team member or a trained treating physician when a critically ill patient is admitted to the ICU, regardless of having a RBC transfusion decision. This procedure, of already having the informed consent instead of asking for informed consent when the decision to transfuse is made, was chosen to minimise the delay of RBC transfusion while waiting for informed consent, in case a blood transfusion is needed at some point during ICU admittance. Participants and legal representatives can always decline study participation at any time for any reason. Data collected up to that point will be used, and no further data will be collected.

Patient and public involvement
The public and patients were not involved in the preparation and design of the study. The results of the study will be disseminated to study participants via a letter after the study has ended.

Eligibility criteria
Critically ill patients with anaemia with an arterial catheter in situ receiving a single RBC unit to correct a Hb threshold of 10 g/dL or lower are included. Patients younger than 18 years, in need of an emergency RBC transfusion, without a legal representative, with a brown plaster allergy, with photodermatosis and/or porphyria or pregnant women are excluded. Furthermore, patients with expected admittance <24 hours, without a central venous catheter in situ and without sufficient Dutch language comprehensibility are excluded. Due to logistics, patients can only be included during working hours (08:00 to 18:00) from Monday to Friday.

Study procedures
In the Leiden University Medical Center, the local transfusion guideline states that transfusion should be guided by the Hb concentration, American Society of Anesthesiologist Classification, age of patient, cardiac state, pulmonary state and volemic state, resulting in varying transfusion thresholds between 6.5 g/dL and 9.7 g/dL. In the Amsterdam University Medical Center and Erasmus University Medical Center Rotterdam, the local transfusion guideline states that an Hb threshold below 7 g/dL should be used in all patients. Treating physicians immediately inform the investigators when they decide to give RBC transfusion to critically ill patients with anaemia during working hours. After assessment of eligibility criteria by the investigator and presence of informed consent, the patient is included in the study. The anterior chest wall of the patient is cleaned with alcohol, and a 5-aminolevulinic acid (ALA) containing patch (Alacare, Photonamic GmbH, Wedel, Germany) is placed on the sternum. The RBC transfusion is given 4 hours after administration of this patch to secure that enough PpIX is formed. In the participating centres, the time between the decision to transfuse and the actual transfusion is between 2 and 3 hours; thus, for patients included in our
to ground state results in emission of delayed fluorescence (650–700 nm), but excited PpIX can transfer its energy to oxygen and fall back to the ground state without emission of light. Due to such oxygen-dependent quenching, the delayed fluorescence lifetime is a measure of mitoPO$_2$, that is, the more oxygen is present, the shorter is the extinction time of the delayed fluorescence signal. With every increase or decrease of skin temperature in degrees Celsius, an increase or decrease in this oxygen-dependent quenching constant is seen. The detection of delayed fluorescence is a delicate process, since the signal in itself is weak, which is overcome by a pulsed excitation with a q-switched laser in combination with time-gated detection. After calculating the delayed fluorescence lifetime from the acquired signal, the mitoPO$_2$ measurement site can be calculated using the Stern-Volmer equation. The necessary calibration constants, the quenching constants, were validated in rat skin, rat liver and rat heart. The measurement location is limited to the epidermis layer since topical ALA skin penetration is possible until a depth of maximum 0.6 mm. Therefore, it is not expected that body fat tissue will affect the mitoPO$_2$ measurement. However, the possible effect of oedema on mitoPO$_2$ measurement cannot be ruled out. There is a risk of PpIX clearance with each measurement, possibly leading to a decline in fluorescence signal, which is also known as photobleaching. However, the COMET uses a light dose less than 2 mJ/cm$^2$ per measurement. Therefore, the influence of PpIX photobleaching on the mitoPO$_2$ measurement, even after repeated measurements, is expected to be negligible and appeared no practical problem in previous studies until now.

**Primary aim**

To assess whether crucial components of the main study—such as the measurement of mitoPO$_2$ in critically ill patients with anaemia—are feasible, we performed a pilot study in 20 critically ill patients with anaemia. There is no non-invasive golden standard of mitoPO$_2$ measurement to compare the COMET performances with. Hence, feasibility is pragmatically determined by the following two parameters: a PpIX-TSLT signal quality $>25\%$ and a within-subject variability $<5\text{ mm Hg}$. Signal quality is measured with the COMET monitor device, in which a higher signal quality corresponds to a stronger PpIX signal and a more sensitive mitoPO$_2$ measurement. Signal-to-noise ratio (SNR) in time domain lifetime measurements (as implemented in the COMET) is commonly defined as the ratio of maximal signal amplitude (at the start of the decay) to the maximum signal of the noise (peak-to-peak). Since SNR is not regarded as an intuitive measure for the end user of the COMET device, the software converts SNR to signal quality using a non-linear relationship in which, for example, an SNR of 100 provides a signal quality of almost 80\%. In a previous study, it was shown that for PpIX-based delayed fluorescence measurements, an SNR $>20$ limits the noise-induced potential error in mitoPO$_2$ measurements below...
2% over the physiological \( \text{PO}_2 \) range. Therefore, we chose to (arbitrarily) define 25% as the minimum required signal quality for a feasible measurement. Within-subject variability is defined as the variation of mitoPO\(_2\) within the subject at each measurement timepoint. The mean mitoPO\(_2\) with its corresponding SD is calculated per participant at each timepoint. The mean SD per timepoint therefore represents the within-subject variability of the study population per timepoint. Pragmatically, a within-subject variability up to 5 mm Hg is defined as a feasible measurement to account for possible measurement errors.

The main aim of the study is to assess the effect of RBC transfusion on mitoPO\(_2\) and to describe the association between mitoPO\(_2\) and traditional parameters used to measure oxygenation, oxygen delivery and oxygen balance (ie, central venous oxygen saturation, arterial oxygen saturation, arterial oxygen tension, central venous-to-arterial carbon dioxide difference, cardiac output and lactate).

**Secondary aims**

Other aims include safety of mitoPO\(_2\) measurements, for which adverse and serious adverse events are recorded, and the description of the association of cutaneous mitoPO\(_2\) with vital organ functions. Vital organ function is assessed by troponin and ischaemic changes on an ECG, 24-hour urine creatinine, Glasgow Coma Scale and Intensive Care Delirium Screening List score and the arterial oxygen tension/fraction inspired oxygen ratio.

**Data collection**

Data is extracted from the hospital’s electronic patient dossier system by the coordinating investigator and coded to ensure anonymity of the patients. Recorded data include age, sex, comorbidities, body mass index, reason of ICU admission and Acute Physiology and Chronic Health Evaluation (APACHE) II\(^{26}\) and IV\(^{26}\) score. The reason of ICU admission is recorded in accordance with the APACHE IV scoring system.\(^{26}\) With each mitoPO\(_2\) measurement, the following parameters are assessed: heart rate, blood pressure, central venous pressure, peripheral oxygen saturation, body temperature, cardiac output, arterial lactate, central venous oxygen saturation, arterial oxygen saturation, arterial oxygen tension and central venous-to-arterial carbon dioxide difference. Cardiac output is assessed by a limited invasive method, the Vigileo/FloTrac system.\(^{26}\) Information regarding use and dosage of noradrenalin, adrenalin, phenylephrine, dobutamine, dopamine, milrinone and enoximone are collected with each mitoPO\(_2\) measurement. In addition, Sequential Organ Failure Assessment (SOFA) score,\(^{29}\) the need for mechanical ventilation and the amount of fractional inspired oxygen on the day of transfusion and 1 day after transfusion are also recorded. Data regarding RBC transfusion are recorded as well, including timing of RBC transfusion, infusion duration, number of RBC transfusions, history of RBC transfusions and product code of the RBC units. Clinical outcome data are extracted from the electronic health record in the 3-month follow-up time and include ICU length of stay, hospital length of stay, ICU mortality and in-hospital mortality. Critically ill patients will be observed during study participation and afterwards for the possible side effects of the ALA plaster. We will collect and report data regarding adverse events, serious adverse events and suspected unexpected serious adverse reactions to gain insight in the safety of the mitoPO\(_2\) measurement.

**Missing data**

After the last recruitment, data will be reviewed to check for outliers and missing data. Multiple imputation will be used to handle missing data.\(^{30}\)

**Statistical analysis of the pilot study**

Descriptive statistics was used to describe the characteristics of the study population. The mitoPO\(_2\) at the different timepoints before and after transfusion were described with medians and IQRs or as means with SD depending on the distribution of the data. For each timepoint, the mean of all five measurements of mitoPO\(_2\) was calculated with a corresponding SD for each patient. These patient-specific means were used to calculate the population mean at each timepoint. The SDs of the patient-specific means at each timepoint were used to calculate the within-subject variability, which is the mean of these SDs at each timepoint. Signal quality of the mitoPO\(_2\) measurement was described using the mean and SD of the signal quality.

**Results of the pilot study**

The pilot study was performed between May and August 2017. During the 3-month study period, 233 RBC transfusions were given to 114 patients (figure 1). After assessment of eligibility criteria, 20 patients were included in the pilot study.

In two study participants, no consecutive mitoPO\(_2\) measurements were possible due to discharge from the ICU. The delayed fluorescence signals were detected in all but one patient who had a large amount of red-coloured chlorhexidine on the chest after cardiothoracic surgery, despite cleaning the skin with alcohol. A large amount of red-coloured chlorhexidine possibly influences the delayed fluorescence detection, thereby leading to a low signal quality. The demographic data of the 18 included patients in the analysis can be found in table 1.

The mean mitoPO\(_2\) before start of RBC transfusion was 70.5 mm Hg (SD 13 mm Hg), which largely corresponded with the mean mitoPO\(_2\) value in healthy volunteers (mean 66.3 mm Hg, SD 16 mm Hg).\(^{11}\) Since there is no known normal range of mitoPO\(_2\) interpretation needs to be performed cautiously. Reliable signals were detectable in all remaining patients (n=18), with a signal quality above 25% during all the measurements (table 2). The within-subject variability of mitoPO\(_2\) was 3.0 mm Hg.
Figure 1  Flow diagram of enrolment of study participant for the pilot study of the INOX ICU-2 study. ICU, intensive care unit; INOX-ICU-2, Insufficient OXygenation in ICU patients-2; RBC, red blood cell.

Before RBC transfusion, 5.8 mm Hg 30 min after RBC transfusion, 3.6 mm Hg 2 hours after RBC transfusion, 7.3 mm Hg 3 hours after RBC transfusion and 8.7 mm Hg 24 hours after transfusion (table 3). No adverse or serious adverse events regarding the ALA patch were observed by the patient, family, nurse, investigators or physician during the mitoPO2 measurements and 1 day after the measurements.

The within-subject variability in this study seems to tally with the observed within-subject variability in healthy volunteers.11 However, there were no data on the course of within-subject variability over time in the healthy volunteers. The observed increase in the within-subject variation in mitoPO2 values over time may have been due to decreased measurement sensitivity, despite having adequate signal quality. Alternatively, other interventions that were performed besides a RBC transfusion in critically ill patients may have contributed to the variation. To assess this further, a separate study will assess the 24-hour course of mitoPO2 in healthy volunteers and in critically ill patients who receive minimal ICU interventions and no RBC transfusion.

Sample size estimation
To the best of our knowledge, no previous data on mitoPO2 values in critically ill patients with anaemia were available. We used data from our pilot study among 20 subjects to calculate the mean and SD of mitoPO2 in the target population to subsequently calculate the sample size. With a p value for statistical significance of 5% and a statistical power of 90%, a mean mitoPO2 of 67.9 mm Hg

| Characteristic                              | Values are median (IQR) or number (%) |
|---------------------------------------------|---------------------------------------|
| Age in years, mean±SD                       | 65.3±13.9                             |
| Male sex                                    | 13 (72.2)                             |
| BMI, mean±SD                                 | 26.3±4.2                              |
| Admission reason (according to APACHE IV)   |                                       |
| Surgical                                    | 7 (38.9)                              |
| System                                      | 1 (14.3)                              |
| Cardiovascular                              | 6 (85.7)                              |
| Respiratory                                 | 0 (0)                                 |
| Digestive                                   | 0 (0)                                 |
| Neurosurgery                                | 0 (0)                                 |
| Genitourinary                               | 0 (0)                                 |
| Trauma                                      | 0 (0)                                 |
| Miscellaneous                               | 0 (0)                                 |
| Non-surgical                                | 11 (61.1)                             |
| Cardiovascular                              | 1 (9.1)                               |
| Respiratory                                 | 5 (45.5)                              |
| Sepsis                                      | 3 (27.3)                              |
| Neurological                                | 1 (9.1)                               |
| Miscellaneous                               | 1 (9.1)                               |
| Chronic comorbidities (according to APACHE IV) | 8 (44.4)                             |
| Chronic cardiovascular insufficiency         | 0 (8.3)                               |
| Chronic obstructive lung disease            | 1 (8.3)                               |
| Chronic respiratory insufficiency           | 1 (8.3)                               |
| Chronic renal insufficiency                 | 1 (8.3)                               |
| Haematological malignancy                   | 4 (33.3)                              |
| Immunological deficiency                    | 2 (16.7)                              |
| Diabetes mellitus                           | 3 (25)                                |
| Days already admitted to ICU                | 4 (2–11)                              |
| APACHE II score                             | 23.5 (20–30)                          |
| APACHE IV score                             | 86 (73–103)                           |
| SOFA score                                  | 8 (4–11)                              |
| Baseline Hb in g/dL                         | 7.7 (7.3–7.9)                         |
| Baseline Ht in L/L                          | 0.237 (0.220–0.246)                   |
| Number of patients using vasopressors       | 9 (50)                                |
| Number of patients with a central venous catheter in place | 16 (88.9)                             |
| Number of red cell transfusions             | 1.0 (1–1)                             |
| Number of patients receiving RBC before inclusion | 16 (88.9)                             |

Continued
and a SD of 13 mm Hg, we need 150 subjects to show a change in mitoPO₂ of at least 13 mm Hg after RBC transfusion, taking into account an expected 10% attrition rate.

### Statistical analysis of the main study

Descriptive statistics will be used to describe the characteristics of the study population. The following baseline characteristics of the study population will be shown: age in years, sex, body mass index, reason of admission characteristics of the study population. The following baseline descriptive statistics will be used to describe the characteristics.

The study will be conducted according to the 1964 Helsinki declaration and its later amendments. Amendments are changes made to the study after a favourable opinion by the accredited institutional ethics committee has been given. All amendments first have to be approved by the institutional ethics committee.

### ETHICS AND DISSEMINATION

#### Institutional review board approval

The institutional ethics committee of each participating centre approved the study (reference P16.303), which is conducted according to the 1964 Helsinki declaration and its later amendments. Amendments are changes made to the study after a favourable opinion by the accredited institutional ethics committee has been given. All amendments first have to be approved by the institutional ethics committee.
committee of Leiden University Medical Center before any changes can be made and communicated to the other participating centres. The latest amendment is study protocol version 5.0 of 27 February 2019, which has been approved by the institutional ethics committee. This research is financially supported by Grant PPOC-16–31 by Sanquin Research, Amsterdam, the Netherlands.

Data handling and dissemination plan
All patients are assigned a random patient identification code after inclusion, which is generated by the electronic case report form Castor Electronic Data Capture. The code book is stored digitally, with restricted access, and encrypted with a password. All handling of personal data complies with the EU General Data Protection Regulation and the Dutch Act on Implementation of the General Data Protection Regulation. Extracted study data will be coded and entered in the electronic case report form Castor and stored for further publication. To ensure data quality, a data dictionary was made before start of the study and implemented digitally in the electronic case report, including range checks. Only the coordinating investigator, the principal investigators, the study monitor and Health and Youth Care Inspectorate will have access to the final dataset. Multiple times a year, the study will be monitored, and source data verification will be applied as depicted in the monitor plan in the study protocol, via the independent monitor pool of Leiden University Medical Center. No data safety monitoring board was set up, as it was deemed unnecessary by an independent expert. The study protocol was published before start of the study on clinicaltrials.gov. The results of the study will be published in (inter-)national scientific journals and guidelines. The International Committee of Medical Journal Editors authorship criteria will be used to define authors in these publications.

Current status of the main study
One of the main results of the pilot study was that measurement of mitoPO₂ levels using the PpIX-TSLT in critically ill patients receiving RBC transfusions was feasible, since the signal quality was above 25% during all timepoints, and the within-subject variability was acceptable in the first 2 hours after RBC transfusion. However, the results of the pilot study led to three changes in the main study design.

First, having a central venous catheter in place was dismissed as an inclusion criterion, due to the slow inclusion rate and no influence on the primary outcome.

At the end of the pilot study, we noticed that multiple patients were not included in the study, due to not having a written informed consent in time (42 out of 114 transfused patients). A second important result of the pilot study was therefore a change in the consent procedure. Previous research had already shown that approximately 10% of critically ill patients can make an autonomous decision regarding research participation, thus most decisions are made by their substitute decision makers who are already overwhelmed by the medical information concerning their relative and/or loved one. Furthermore, studies have also shown that obtaining contact with the substitute decision makers can be difficult, especially in narrow time windows for inclusion.34 35 The same was seen in our study, and this resulted in a low accrual rate and was possibly influencing the generalisability of our study. We noticed that nearly all substitute decision makers decided positively on participation the second day of admittance of their loved one, once the first shock had decreased. We therefore introduced deferred consent in our study. In principal, we still ask all ICU patients, or their substitute, admitted to the ICU and expected to stay at the ICU for longer than 24 hours for participation in our study. However, if no substitute can be reached in person for written informed consent, while the patient meets the eligibility criteria, the patient is included in the study and deferred informed consent is sought within 48 hours after inclusion.

The third important result of the pilot study was the change in the amount of timepoints. Due to logistic problems during the pilot study, the number of timepoints was minimised to 6: before transfusion, at the end of transfusion, 30, 60 and 180 min after transfusion and 24 hours after transfusion. All of these changes were laid down in amendments to the study protocol and reviewed by the institutional review board, which approved these changes. The main study has started in March 2018 and is ongoing. End of recruitment is expected halfway 2020.

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Contributors All authors were involved in the design of the study. MB and MSA collected data in Leiden University Medical Center, EGM in Erasmus Medical Center and NPJ in Amsterdam Medical Center. MB performed the analysis under supervision of MSA and JSvbO. MB drafted the manuscript. All authors reviewed and edited the manuscript. All authors approved the final manuscript.

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Competing interests EGM is founder and shareholder of Photonics Healthcare B.V (Utrecht, The Netherlands). Photonics Healthcare develops and commercialises the COMET measuring system for mitochondrial oxygen measurements. The other authors have no conflicts of interest to declare.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not required.
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