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ProTreat – Prognosis and treatment of necrotizing soft tissue infections: A prospective cohort study

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1. Title

ProTreat – Prognosis and treatment of necrotizing soft tissue infections:
A prospective cohort study

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2. Abstract

Introduction

Not enough is known regarding the prognosis and treatment of necrotizing soft tissue infections (NSTIs). Mortality has been shown to be 25-35 %, with survivors coping with amputations and prolonged rehabilitation. This study will evaluate suPAR as a possible prognostic marker of NSTI severity and mortality, as well as whether hyperbaric oxygen therapy (HBOT) can modulate markers of endothelial damage during NSTI. We hypothesize that in patients with NSTI, suPAR can provide prognostic risk assessment upon hospital admission and that HBOT can reduce the endothelial damage that these patients are exposed to.

Methods and analysis

This is a prospective, observational study. Biomarkers will be measured in 150 patients who have been diagnosed with NSTI. On admission, baseline blood samples will be obtained. Following surgery and HBOT, daily blood samples will be obtained in order to measure endothelial and prognostic biomarkers (soluble thrombomodulin, syndecan-1, sE-Selectin, VE-cadherin, protein C and suPAR levels). Clinical data will be acquired during the first seven days of stay in the intensive care unit. The primary outcomes in studies I and II will be endothelial biomarker levels after HBOT, and in study III suPAR levels as a marker of disease prognosis and severity.

Ethics and dissemination

The study has been approved by the Regional Scientific Ethical Committee of Copenhagen (H-16021845) and the Danish Data Protection Agency (RH-2016- 199). Results will be presented at national and international conferences and published in peer-reviewed scientific journals.

Trial Registration

ClinicalTrials.gov Identifier NCT03147352

Strengths and limitations of this study

Strengths:

- It is a large, prospective cohort study of necrotizing soft tissue infections (NSTI)
- The study will measure biomarkers never previously examined in NSTI patients
- The study will provide valuable evidence for future optimization of prognosis and treatment

Limitations:

- Due to the non-randomized design, we may be subject to various biases

3. Introduction

Necrotizing soft-tissue infections (NSTI) are among the most serious and deadly infections known. They are characterized by rapidly progressing soft-tissue inflammation with necrosis and can quickly cause multiple organ failure and death. They have a wide range of presentations. Patients can become mortally infected in hours. Mortality has been shown to be 25-35 %, with survivors coping with amputations and prolonged rehabilitation (2, 3, 13). Septic shock accompanies death due to NSTI.

Currently, we lack the proper tools to evaluate the severity and prognosis of NSTI in individual patients. This results in necessary, yet sometimes overzealous surgical debridement, culminating in prolonged patient rehabilitation and invalidity. Hyperbaric oxygen therapy (HBOT) may be added as adjunctive therapy of NSTI. However, we lack a clear understanding of the effectiveness of HBOT on NSTI. We seek to remedy these two issues by investigating multiple biomarkers over the course of several studies.

Accordingly, our first and second study will analyze markers of endothelial function in order to examine the effect of HBOT on NSTI patients. Our third study will look at the prognostic value of

soluble urokinase-type plasminogen activator receptor (suPAR) in NSTI. SuPAR is a biomarker reflecting immune system activity.

3.1 Studies I and II: Endothelial function during NSTI and the effects of hyperbaric oxygen therapy

Endothelial dysfunction during sepsis is the result of a reduction of the endothelial glycocalyx, which leads to platelet aggregation, leucocyte adhesion and an increase in endothelial permeability. The result is capillary leakage and tissue edema. At the same time, the patients' blood is anticoagulated endogenously, which ultimately contributes to intravascular volume depletion. Tissue dysfunction is due to inflammation, reduced tissue blood flow and ischemia, which can lead to multi-organ failure and death (11-14). Recently, we have demonstrated in more than 4.400 patients with acute critical illness (sepsis (16-18), trauma (19), myocardial infarction (20) and resuscitated cardiac arrest (21)) that endothelial breakdown as evaluated by the biomarkers soluble thrombomodulin (sTM) and syndecan 1 is independently associated with development of multi-organ failure and death. Soluble thrombomodulin and syndecan 1 have been shown as markers of endothelial and glycocalyx damage, respectively (22, 23).

In septic rats, HBOT has been shown to attenuate levels of proinflammatory cytokines and prevent coagulation disorders (1-4). Furthermore, HBOT may improve microcirculation by inducing the formation of reactive oxygen species (ROS) and decreasing the adherence of polymorphonuclear neutrophils to the endothelial cell wall (5-8), possibly by downregulation of intracellular adhesion molecule-1 (ICAM-1) (9,10). sE-Selectin and VE-cadherin are markers of leukocyte adhesion and endothelial barrier function, respectively (24, 25).

We believe it is plausible to consider the potential beneficial effects of HBOT on NSTI patients in septic shock due to HBOT mediating an endothelial/glycocalyx protective effect, which enhances the endothelial integrity with its effects on coagulation and platelet reactivity and functionality (6-10). Also, HBOT has been shown to induce a cytoprotective and angiogenic response in human endothelial cells (15).

Therefore, the purpose of studies I and II will be to investigate the effect of HBOT on possible endothelial dysfunction in patients with NSTI. We will do this by measuring sTM and syndecan-1 in study I, as well as sE-Selectin, VE-cadherin and protein C in study II.

3.2 Study III – suPAR as a prognostic biomarker for NSTI

Soluble urokinase-type plasminogen activator receptor has been shown to predict the risk of developing a wide range of chronic conditions, as well as predicting mortality during acute conditions. The risk of developing cardiovascular disease, diabetes mellitus, cancer (26, 27), acute exacerbation of chronic obstructive lung disease (28), mortality during bacteraemia (29,30), mortality during bacterial meningitis (31), mortality from systemic inflammatory response syndrome (SIRS)(32) as well as negative prognosis during sepsis (33) are all correlated with higher than normal levels of suPAR.

In our third study, we will assess suPARs possible value as a prognostic biomarker for mortality and morbidity as well as clinical condition during NSTI.

4. Methods and analysis

4.1 Study design

The ProTreat study is a prospective, observational substudy of the INFECT project (clinicaltrials.gov Identifier: NCT01790698). The INFECT project involves five centres (Copenhagen University Hospital, Karolinska Institute, Blekinge Hospital, Sahlgrenska Hospital, University Hospital of Bergen) with the objective of improving the outcome in patients with NSTI.

The ProTreat study will be conducted at Copenhagen University Hospital and Hvidovre Hospital. The study will use data gathered in Denmark on patients diagnosed with NSTI, who are admitted to Copenhagen University Hospital. The patients are enrolled immediately upon diagnosis using predefined criteria for NSTI, as specified below. The first patient was enrolled on the 26th of February 2013 and inclusion is ongoing. Due to the low incidence of NSTI, the enrolment period of this study will extend over four and a half years, with expected closure by the end of August 2017.

For study III, only the NSTI patient cohort will be used. For studies I and II, we will also use data gathered from a group of 65 elective orthopedic surgery patients, functioning as controls for our NSTI patients. Furthermore, data on endothelial function from the Scandinavian Starch for Severe Sepsis/Septic Shock (6S) trial (clinicaltrials.gov: NCT00962156) of sepsis patients will be used to illustrate and compare with the modulation of endothelial function in sepsis patients who do not receive HBOT.

4.2 Inclusion and exclusion criteria

Study I, II and III: NSTI patients

Patient inclusion criteria are (all of which must be met):

1) Diagnosed with NSTI based on surgical findings (necrosis of any soft tissue compartment; dermis, hypodermis, fascia or muscle)

2) Age ≥18 years

3) Admitted to the Intensive Care Unit (ICU) and/or operated for NSTI at Copenhagen University Hospital.

Patient exclusion criteria are:

1) They are categorized as non NSTI in the operating theatre

Studies I and II: Orthopedic control patients

Control patient inclusion criteria are (all of which must be met):

1) Undergoing elective orthopedic surgery (non-pathological fractures, joint replacement surgery or spine surgery) at Copenhagen University Hospital

2) Age ≥18 years

Patient exclusion criteria are:

1) Ongoing infection or inflammatory condition

4.3 Data collection

A blood sample taken from an arterial line from each NSTI patient is collected into tubes containing EDTA at four time points: Once upon admission and once each of the following three days, always between 0800 and 1400. During the first seven days in the ICU, clinical data will be gathered. For the orthopedic control group, the blood samples have been drawn at three time points: once at baseline (preoperatively), once two to six hours postoperatively and once on the day after surgery between 0800 and 1200. For both patient groups, the anticoagulated blood is put on ice until centrifugation (within 40 minutes of collection, at 3500 rpm for 10 minutes). The supernatant (serum) is stored in 1 mL vials at −80°C until analysis.

Table 1

Overview of blood sampling procedures

Day 0 (time of admission)	Day 1	Day 2	Day 3
NSTI patients	*NSTI patients*	*NSTI patients*	*NSTI patients*
EDTA blood (2 collection tubes)	EDTA blood (2 collection tubes)	EDTA blood (2 collection tubes)	EDTA blood (2 collection tubes)

- **NSTI, necrotizing soft tissue infection**

Table 2

Baseline characteristics and clinical data

Data	Description
Baseline characteristics	▸ Sex and age ▸ Comorbidities – Diabetes mellitus, cirrhosis of the liver, renal disease, heart disease, vascular disease, hepatitis, IV drug abuse, history of cancer, COPD, immunosuppression ▸ Body mass index ▸ Primary infection site ▸ Origin of infection – chronic wound, injection, boil/furuncle, animal bite, idiopathic, trauma, postoperative infection, perianal abscess, other ▸ Symptoms registered at the primary hospital – edema, erythema, tachycardia, fever, bullae ▸ Responsible microorganism ▸ Time between admission to primary hospital and first debridement ▸ Time between admission to primary hospital and admission to ICU ▸ Steroid treatment (injection/oral) prior to development of NSTI [Time Frame: Up to 7 days prior to surgical diagnosis at primary hospital] ▸ Other medication
Clinical data from the ICU	▸ MAP (mm Hg) ▸ Heart rate (bpm) ▸ Arterial blood gas values: pO_2, pCO_2, HCO_3^-, base excess, pH ▸ K^+, Na^+, Ca^{2+}, glucose, creatinine, hemoglobin, hematocrit ▸ Norepinephrine infusion ▸ Ventilator treatment

Data	Description
	▸ Vasopressor treatment ▸ Renal replacement treatment ▸ LRINEC score

- **COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; IV, intravenous; LRINEC, Laboratory Risk Indicator for Necrotising Fasciitis; MAP, mean arterial blood pressure**

4.4 Data analysis

Study I, II and III: Routine blood analysis

These tests will be run at the Department of Clinical Biochemistry, Copenhagen University Hospital. Among others: platelets, pH, base excess, fibrinogen, INR, D-dimer, CRP, procalcitonin, lactate, bilirubin, potassium, sodium, calcium, glucose, creatinine, haemoglobin, leucocytes.

Study I and II: sTM, syndecan-1, sE-Selectin, VE-cadherin and protein C levels

These tests will be conducted at the Department of Clinical Immunology, Copenhagen University Hospital. All the biomarkers will be measured using ELISA methods from various companies (Nordic Biosite for sTM, syndecan-1 and sE-Selectin; R&D Systems for VE-Cadherin; Orion Diagnostica for protein C).

Study III: suPAR levels

These tests will be conducted at the Clinical Research Department, Hvidovre Hospital. SuPAR levels will be measured using ELISA from ViroGates. It is based on a simplified double monoclonal antibody sandwich ELISA assay, whereby samples and peroxidase-conjugated anti-suPAR are first mixed together and then incubated in anti-suPAR pre-coated micro wells. The recombinant suPAR standards of the kit are calibrated against healthy human blood donor samples. suPAR concentrations are determined as ng/mL plasma.

4.5 Hypotheses; primary and secondary outcomes

Study I

Study I hypotheses

1) In NSTI patients stratified into no sepsis, sepsis, severe sepsis and septic shock groups, two hours of HBOT at 1.8 ATA lowers sTM more than 1.75 ng/ml per day.

2) The aforementioned reduction in sTM and/or syndecan-1 in NSTI patients after HBOT is statistically significantly larger than any reduction in sTM and/or syndecan-1 seen in both an elective orthopedic surgery control group and sepsis control group.

Study I primary outcome

Changes in plasma sTM and syndecan-1 concentrations, measured once upon admission and once daily the first three days in the ICU.

Study I secondary endpoint

A sub-analysis of the differences in the aforementioned endothelial biomarkers between NSTI patients who do not receive HBOT within the first 24 hours of ICU admission (because they are deemed too unstable for HBOT) vs. those who receive HBOT within the first 12 and 24 hours of ICU admission

Study II

Study II hypothesis

1. In NSTI patients stratified into no sepsis, sepsis, severe sepsis and septic shock groups, two hours of HBOT at 1.8 ATA lowers sE-selectin more than 1.1 ng/ml per day.
2. The aforementioned reduction in sE-selectin in NSTI patients after HBOT is statistically significantly larger than any reduction in sE-selectin seen in both an elective orthopedic surgery control group and sepsis control group.

Study II primary endpoints

Changes in plasma sE-selectin, VE-cadherin and Protein C concentrations, measured once upon admission and once daily the first three days in the ICU.

Study II secondary endpoint

A sub-analysis of the differences in the aforementioned biomarkers between NSTI patients who do not receive HBOT within the first 24 hours of ICU admission (because they are deemed too unstable for HBOT) vs. those who receive HBOT within the first 12 and 24 hours of ICU admission

Study III

Study III hypotheses

In NSTI patients stratified into no sepsis, sepsis, severe sepsis and septic shock groups, suPAR levels are a predictor for mortality.

In NSTI patients stratified into no sepsis, sepsis, severe sepsis and septic shock groups, suPAR levels reflect NSTI patients' clinical condition as assessed by SAPS II and SOFA scores.

Study III primary endpoint

Association between plasma suPAR levels, measured once upon admission and once daily during the first three days in the ICU, and NSTI mortality, SAPS II and SOFA scores.

Joint secondary endpoints for studies I, II and III

In study I, II and III, the following outcomes will be analyzed for the NSTI group only:

- Mortality at ICU, in-hospital and at 28, 90, 180 days as well as long term of up to 4 years
- Amputations

The following characteristics are registered in the INFECT database, which we will also be using for our studies:

- Age and sex
- Comorbidities: diabetes mellitus, liver cirrhosis, kidney disease, cardiovascular disease, HIV/AIDS, hepatitis, intravenous drug use, malignancy
- Body mass index (BMI)
- Middle arterial pressure (MAP)
- Heart rate
- Arterial blood gas: pO_2, pCO_2, HCO_3, base excess, pH
- Standard biochemistry: K^+, Na^+, Ca2+, glucose, creatinine, haemoglobin etc.
- Norepinephrine use
- Mechanical ventilation

- ICU scores: LRINEC SCORE, SAPS II, APACHE, SOFAS without GCS, MODS
- Primary infectious focus
- Primary symptoms: pain, erythema, tachycardia, fever
- Pathogen type
- Time between admittance at primary hospital to the first surgery
- Definitive treatment at Copenhagen University Hospital: antibiotics, immunoglobuline and HBOT
- Treatment at primary hospital: antibiotics, immunoglobuline, surgical treatment
- Immunocompromising drugs prior to admission

4.6 Sample size

Study I

The test kits we will be using to measure our primary outcome sTM (Human sCD141 ELISA kit, *Nordic Biosite*) have an interassay standard variation of 0.58 ng/ml. In order to be certain that measured changes in sTM concentration are not a result of interassay standard deviation, we have set our mimimum relevant difference in sTM to 3 x the interassay standard variation, thus 1.75 ng/ml.

We prepared a power calculation using a Wilcoxon rank sum test. Assuming an estimated standard deviation of 4.6 ng/ml and a mean of 9.9 ng/ml (18), we will need to include a maximum of 150 NSTI patients and 50 elective surgery patients to reach a statistical power of at the very least 60 % (a very conservative estimate) and presumably closer to 85 % (more realistic estimate) at a 5 % significance level. The estimates depend on data distribution.

Study II

The test kits we will be using to measure our primary outcome sE-selectin (Human CD62E ELISA kit, *Diaclone*) have an interassay standard variation of 0.37 ng/ml. In order to be certain that measured changes in sE-selectin concentration are not a result of interassay standard variation, we have set our mimimum relevant difference in sE-selectin to 3 x the interassay standard variation, thus 1.1 ng/ml.

Assuming an estimated standard deviation of 209 ng/ml (septic shock) vs. 23 ng/ml (severe sepsis and sepsis) and means of 295 vs. 181 ng/ml, respectively (36), we will need to include at least 132

NSTI patients and 50 elective surgery patients to reach a statistical power of 90 % at a 5 % significance level.

Study III

suPAR levels during NSTI have never previously been examined. In order to estimate sample size and since NSTI patients are also septic, we are basing our sample size calculation on a previous study concerning the correlation between suPAR and sepsis (9). This study found statistically significant correlation between suPAR levels and mortality in 141 patients. This is also our goal. Further studies have also found significant correlations between suPAR, sepsis and mortality in 132 patients (10). We will include at least 150 NSTI patients during this study.

4.7 Statistical considerations

Studies I and II

To check whether the HBOT treatment has an effect on the range of biomarkers, we will analyze the means and variances of the biomarkers in the NSTI group and the two control groups, the orthopaedic patients and the sepsis patients. Non-parametric data will be log-transformed and will be presented as median values with IQR. Wilcoxon rank sum tests will be used for group comparisons. Fisher's exact test will be used for categorical data. Correlation analysis will be performed using Spearman rank correlation or Pearson correlation.

Study III

To assess the quality of suPAR as a predictor of health outcomes, a model selection exercise will be conducted with various types of regression models. The type of regression will vary with the type of health-outcome, with suPAR as the predictor in all cases. Non-parametric data will be log-transformed and will be presented as median values with IQR. Fisher's exact test will be used for categorical data. Receiver operating characteristic (ROC) curve analysis will be applied to determine suPARs accuracy as a marker of severity and mortality in patients with NSTI. We will construct Kaplan-Meier curves for survival data. Statistically significant results are when $p<0.05$. Corrections for multiple comparisons will be done using Wilcoxon rank sum (HDR) tests.

4.8 Ethics and dissemination

The study will be conducted in accordance with the principles of the Declaration of Helsinki. The Regional Health Research Ethics Committee and the Danish Data Protection Agency (responsible

for the correct processing of confidential patient data) have approved the study (RHREC doc. nr.: H-16021845; DDPA j.nr.: RH-2016- 199). The investigator will inform the Research Ethics Committee and the Danish Data Protection Agency of any significant changes to the protocol.

Written informed consent will be acquired from either the patients themselves or their next of kin as well as from their primary healthcare physician, as required by Danish law. This study itself poses no additional risk to the patients, as patients will receive standard NSTI treatment at Copenhagen University Hospital, in no way different from the usual treatment. To maintain confidentiality, each patient is assigned a pseudonymous research code. Access to patient data analysis is restricted to the investigators.

The study has been registered at the international database of clinical trials at www.clinicaltrials.gov NCT03147352.

Results will be disseminated at national and international conferences and then published in international peer-reviewed scientific journals. Positive, negative and any inconclusive results will be published. The advanced knowledge of NSTIs generated by the above studies will be used to create evidence based guidelines for classification and management. Through the INFECT project, we have access to the UK NSTI patient organization together with the NSTI clinical consortium. This provides excellent means for efficient dissemination of guidelines and other advances made in the project to relevant end-users, including medical staff, patients and their relatives, SMEs and researchers.

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4.9 Authors' contributions

PP and OH designed and wrote the research protocol. PP is responsible for the sample size calculations and statistical methods. PP is responsible for data acquisition. PJ contributed to drafting the protocol. PJ is responsible for the laboratory work in study I and II.

4.10 Funding

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4.11 Competing interests

None declared.

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How biomarkers reflect the prognosis and treatment of necrotizing soft tissue infections and the effects of hyperbaric oxygen therapy: The protocol of the prospective cohort PROTREAT study conducted at a tertiary hospital in Copenhagen, Denmark

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1. Title

How biomarkers reflect the prognosis and treatment of necrotizing soft tissue infections and the effects of hyperbaric oxygen therapy:
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2. Abstract

Introduction

Not enough is known regarding the prognosis and treatment of necrotizing soft tissue infections (NSTIs). Mortality has been shown to be 25-35 %, with survivors coping with amputations and prolonged rehabilitation. This study will evaluate soluble urokinase-type plasminogen activator receptor (suPAR) as a possible prognostic marker of NSTI severity and mortality, as well as whether hyperbaric oxygen therapy (HBOT) can modulate markers of endothelial damage during NSTI. We hypothesize that in patients with NSTI, suPAR can provide prognostic risk assessment upon hospital admission and that HBOT can reduce the endothelial damage that these patients are exposed to.

Methods and analysis

This is a prospective, observational study. Biomarkers will be measured in 150 patients who have been diagnosed with NSTI. On admission, baseline blood samples will be obtained. Following surgery and HBOT, daily blood samples will be obtained in order to measure endothelial and prognostic biomarkers (soluble thrombomodulin, syndecan-1, sE-Selectin, VE-cadherin, protein C and suPAR levels). Clinical data will be acquired during the first seven days of stay in the intensive care unit. The primary outcomes in studies I and II will be endothelial biomarker levels after HBOT, and in study III suPAR levels as a marker of disease prognosis and severity.

Ethics and dissemination

The study has been approved by the Regional Scientific Ethical Committee of Copenhagen (H-16021845) and the Danish Data Protection Agency (RH-2016- 199). Results will be presented at national and international conferences and published in peer-reviewed scientific journals.

Trial Registration

ClinicalTrials.gov Identifier NCT03147352

Strengths and limitations of this study

Strengths:

- It is the largest, single-centre prospective cohort study of biomarkers during necrotizing soft tissue infections (NSTI)
- The study will measure biomarkers never previously examined in NSTI patients
- The study's outcomes may provide valuable evidence for future studies of optimization of NSTI prognosis and treatment

Limitations:

- Due to the non-randomized design, we may be subject to biases due to differences in HBOT allocation and result interpretation

3. Introduction

Necrotizing soft-tissue infections (NSTI) are serious and deadly. They are characterized by rapidly progressing soft-tissue inflammation with necrosis and can quickly cause multiple organ failure and death. They have a wide range of presentations. Patients can become mortally infected in hours. Mortality has been shown to be 25-35 %, with survivors coping with amputations and prolonged rehabilitation (1). Septic shock accompanies death due to NSTI.

Currently, we lack the proper tools to evaluate the severity and prognosis of NSTI in individual patients. This results in necessary, yet sometimes overzealous surgical debridement, culminating in prolonged patient rehabilitation and amputations. Hyperbaric oxygen therapy (HBOT) may be added as adjunctive therapy of NSTI (2, 3, 4). Large database surveys indicate that HBOT improves survival of patients with NSTI in hospitals capable of providing HBOT – the effect being most prominent for severely ill patients, with septic shock (2, 3, 4). Large randomized controlled trials (RCTs) are lacking, in large part due to ethical concerns. However, in the present prospective cohort, HBOT is already being used as part of the standard NSTI treatment in a multidisciplinary setting in a tertiary hospital, with centralized treatment expertise and an in-hospital HBOT unit. We wish to use this unique opportunity to examine the effects of HBOT during NSTI by means of

biomarkers, in order to obtain pathophysiological knowledge about the effects of HBOT. The data will also contribute to improved decision making with respect to the proper design and ethical justification of future RCT studies on the effects of HBOT.

Accordingly, our first and second study will analyze markers of endothelial function in order to examine the effect of HBOT on NSTI patients. Our third study will look at the prognostic value of soluble urokinase-type plasminogen activator receptor (suPAR) in NSTI. SuPAR is a biomarker reflecting immune system activity (5).

3.1 Studies I and II: Endothelial function during NSTI and the effects of hyperbaric oxygen therapy

Endothelial dysfunction during sepsis is the result of damage to the endothelial glycocalyx, which leads to platelet aggregation, leucocyte adhesion and an increase in endothelial permeability. The result is capillary leakage and tissue edema. At the same time, the patients' blood is anticoagulated endogenously. This capillary leakage and anticoagulation ultimately lead to intravascular volume depletion (6-9). Tissue dysfunction is due to inflammation, reduced tissue blood flow and ischemia, which can lead to multi-organ failure and death (6-9). Recently, we have demonstrated in more than 4.400 patients with acute critical illness (sepsis (10-12), trauma (13), myocardial infarction (14) and resuscitated cardiac arrest (15)) that endothelial breakdown as evaluated by the biomarkers soluble thrombomodulin (sTM) and syndecan 1 is independently associated with development of multi-organ failure and death. We are interested in examining whether this also is the case with NSTIs, since most of these patients are also septic. Soluble thrombomodulin and syndecan 1 have been shown as markers of endothelial and glycocalyx damage, respectively (16, 17). sTM is released from endothelial cells upon damage, while damage to the glycocalyx releases syndecan-1. Increases in these markers therefore correspond to increased levels of endothelial damage (18).

In septic rats, HBOT has been shown to attenuate levels of proinflammatory cytokines and prevent coagulation disorders (19-22). Furthermore, HBOT may improve microcirculation by inducing the formation of reactive oxygen species (ROS) (23, 24) and decreasing the adherence of polymorphonuclear neutrophils to the endothelial cell wall (25-28), possibly by downregulation of intracellular adhesion molecule-1 (ICAM-1) (29, 30). sE-Selectin and VE-cadherin are markers of leukocyte adhesion and endothelial barrier function, respectively (31, 32). sE-Selectin is responsible for interactions between leukocytes and the endothelium, and increased expression is

due to endothelial activation (33). Lower concentrations of vE-Cadherin result in loss of vascular integrity (34).

We believe it is plausible to consider the potential beneficial effects of HBOT on NSTI patients in septic shock due to HBOT mediating an endothelial/glycocalyx protective effect, which enhances the endothelial integrity with its effects on coagulation and platelet reactivity and functionality (26-30). Also, HBOT has been shown to induce a cytoprotective and angiogenic response in human endothelial cells (35). A deeper understanding of endothelial dysfunction during NSTI, and the possible countering effect of HBOT, could contribute to a better understanding of this disease.

Therefore, the purpose of studies I and II will be to investigate the effect of HBOT on possible endothelial dysfunction in patients with NSTI. We will do this by measuring sTM and syndecan-1 in study I, as well as sE-Selectin, VE-cadherin and protein C in study II.

3.2 Study III – suPAR as a prognostic biomarker for NSTI

Soluble urokinase-type plasminogen activator receptor has been shown to predict the risk of developing a wide range of chronic conditions, as well as predicting mortality during acute infectious conditions. The risk of developing cardiovascular disease, diabetes mellitus, cancer (36, 37), acute exacerbation of chronic obstructive lung disease (38), mortality during bacteraemia (39-41), mortality during bacterial meningitis (42), mortality from systemic inflammatory response syndrome (SIRS) (43) as well as negative prognosis during sepsis (44) are all correlated with higher than normal levels of suPAR. Likewise, since NSTIs are also infectious diseases, we are interested in examining suPARs potential during NSTIs.

In our third study, we will assess suPARs possible value as a prognostic biomarker for mortality and morbidity as well as clinical condition during NSTI.

4. Methods and analysis

4.1 Study design

The PROTREAT study is a prospective, observational substudy of the INFECT project (clinicaltrials.gov Identifier: NCT01790698). The INFECT project involves five centres (Copenhagen University Hospital, Karolinska Institute, Blekinge Hospital, Sahlgrenska Hospital, University Hospital of Bergen) with the objective of improving the outcome in patients with NSTI.

The PROTREAT study will be conducted at Copenhagen University Hospital and Hvidovre Hospital. The study will use data gathered in Denmark on patients diagnosed with NSTI, who are admitted to Copenhagen University Hospital. The patients are enrolled immediately upon diagnosis using predefined criteria for NSTI, as specified below. The first patient was enrolled on the 26th of February 2013 and inclusion is ongoing. Due to the low incidence of NSTI, the enrolment period of this study will extend over four and a half years, with expected closure by the end of August 2017.

For study III, only the NSTI patient cohort will be used. For studies I and II, we will also use data gathered from a group of 65 elective orthopedic surgery patients, functioning as controls for our NSTI patients. Furthermore, data on endothelial function from the Scandinavian Starch for Severe Sepsis/Septic Shock (6S) trial (clinicaltrials.gov: NCT00962156) of sepsis patients will be used to illustrate and compare with the modulation of endothelial function in sepsis patients who do not receive HBOT.

4.2 Inclusion and exclusion criteria

Study I, II and III: NSTI patients

Patient inclusion criteria are (all of which must be met):

1) Diagnosed with NSTI based on surgical findings (necrosis of any soft tissue compartment; dermis, hypodermis, fascia or muscle)

2) Age ≥18 years

3) Admitted to the Intensive Care Unit (ICU) and/or operated for NSTI at Copenhagen University Hospital.

Patient exclusion criteria are:

1) They are categorized as non NSTI in the operating theatre

Studies I and II: Orthopedic control patients

Control patient inclusion criteria are (all of which must be met):

1) Undergoing elective orthopedic surgery (non-pathological fractures, joint replacement surgery or spine surgery) at Copenhagen University Hospital

2) Age ≥18 years

Patient exclusion criteria are:

1) Ongoing infection or inflammatory condition

4.3 Data collection

A blood sample taken from an arterial line from each NSTI patient is collected into tubes containing ethylenediaminetetraacetic acid (EDTA) at four time points: Upon admission and each of the following three days, always between 0800 and 1400 (see Table 1). During the first seven days in the ICU, clinical data will be gathered (see Table 2). For the orthopedic control group, the blood samples have been drawn at three time points: once at baseline (preoperatively), once two to six hours postoperatively and once on the day after surgery between 0800 and 1200. For both patient groups, the anticoagulated blood is put on ice until centrifugation (within 40 minutes of collection, at 3500 rpm for 10 minutes). The supernatant (serum) is stored in 1 mL vials at −80°C until analysis.

Table 1

Overview of blood sampling procedures

Day 0 (time of admission)	Day 1	Day 2	Day 3
NSTI patients	*NSTI patients*	*NSTI patients*	*NSTI patients*
EDTA blood (2 collection tubes)	EDTA blood (2 collection tubes)	EDTA blood (2 collection tubes)	EDTA blood (2 collection tubes)

- **NSTI, necrotizing soft tissue infection**

NSTI: necrotizing soft tissue infection; EDTA: ethylenediaminetetraacetic acid

Table 2

Baseline characteristics and clinical data

Data	Description
Baseline characteristics	▸ Sex and age ▸ Comorbidities – Diabetes mellitus, cirrhosis of the liver, renal disease, heart disease, vascular disease, hepatitis, IV drug abuse, history of cancer, COPD, immunosuppression

Data	Description
	▸ Body mass index ▸ Primary infection site ▸ Origin of infection – chronic wound, injection, boil/furuncle, animal bite, idiopathic, trauma, postoperative infection, perianal abscess, other ▸ Symptoms registered at the primary hospital – edema, erythema, tachycardia, fever, bullae ▸ Responsible microorganism ▸ Time between admission to primary hospital and first debridement ▸ Time between admission to primary hospital and admission to ICU ▸ Steroid treatment (injection/oral) prior to development of NSTI [Time Frame: Up to 7 days prior to surgical diagnosis at primary hospital] ▸ Other medication
Clinical data from the ICU	▸ MAP (mm Hg) ▸ Heart rate (bpm) ▸ Arterial blood gas values: pO_2, pCO_2, HCO_3^-, base excess, pH ▸ K^+, Na^+, Ca^{2+}, glucose, creatinine, hemoglobin, hematocrit ▸ Norepinephrine infusion ▸ Ventilator treatment ▸ Vasopressor treatment ▸ Renal replacement treatment ▸ LRINEC score

- **COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; IV, intravenous; LRINEC, Laboratory Risk Indicator for Necrotising Fasciitis; MAP, mean arterial blood pressure; NSTI, necrotizing soft tissue infection**

4.4 Data analysis

Study I, II and III: Routine blood analysis

These tests will be run at the Department of Clinical Biochemistry, Copenhagen University Hospital. Among others: platelets, pH, base excess, fibrinogen, INR, D-dimer, CRP, procalcitonin, lactate, bilirubin, potassium, sodium, calcium, glucose, creatinine, haemoglobin, leucocytes.

Study I and II: sTM, syndecan-1, sE-Selectin, VE-cadherin and protein C levels

These tests will be conducted at the Department of Clinical Immunology, Copenhagen University Hospital. All the biomarkers will be measured using ELISA methods from various companies

(Nordic Biosite for sTM, syndecan-1 and sE-Selectin; R&D Systems for VE-Cadherin; Orion Diagnostica for protein C).

Study III: suPAR levels

These tests will be conducted at the Clinical Research Department, Hvidovre Hospital. SuPAR levels will be measured using ELISA from ViroGates. It is based on a simplified double monoclonal antibody sandwich ELISA assay, whereby samples and peroxidase-conjugated anti-suPAR are first mixed together and then incubated in anti-suPAR pre-coated micro wells. The recombinant suPAR standards of the kit are calibrated against healthy human blood donor samples. suPAR concentrations are determined as ng/mL plasma.

4.5 Hypotheses; primary and secondary outcomes

Study I

Study I hypotheses

1) In NSTI patients stratified into no sepsis, sepsis, and septic shock groups as defined by standardized criteria (45), HBOT* lowers sTM more than 1.75 ng/ml per day.
2) The aforementioned reduction in sTM in NSTI patients after HBOT is statistically significantly larger than any reduction in sTM seen in both an elective orthopedic surgery control group and sepsis control group.

* HBOT is applied by placing the NSTI patient inside a HBOT chamber, where the patient is continuously breathing 100 % O_2 through a ventilator and endotracheal intubation or if awake through a transparent hood and where the entire chamber is pressurized to 2.8 atmospheres absolute (ATA) for 90 minutes.

Study I primary outcome

Changes in plasma sTM and syndecan-1 concentrations, measured upon admission and once daily the first three days in the ICU.

Study I secondary endpoint

A sub-analysis of the differences in the aforementioned endothelial biomarkers between NSTI patients who do not receive HBOT within the first 24 hours of ICU admission (because they are deemed too unstable for HBOT) vs. those who receive HBOT within the first 12 and 24 hours of ICU admission

Study II

Study II hypothesis

1. In NSTI patients stratified into no sepsis, sepsis, and septic shock groups as defined by standardized criteria (45), HBOT* lowers sE-selectin more than 1.1 ng/ml per day.
2. The aforementioned reduction in sE-selectin in NSTI patients after HBOT is statistically significantly larger than any reduction in sE-selectin seen in both an elective orthopedic surgery control group and sepsis control group.

* HBOT is applied by placing the NSTI patient inside a HBOT chamber, where the patient is continuously breathing 100 % O_2 through a ventilator and endotracheal intubation or if awake through a transparent hood and where the entire chamber is pressurized to 2.8 atmospheres absolute (ATA) for 90 minutes.

Study II primary endpoints

Changes in plasma sE-selectin, VE-cadherin and Protein C concentrations, measured upon admission and once daily the first three days in the ICU.

Study II secondary endpoint

A sub-analysis of the differences in the aforementioned biomarkers between NSTI patients who do not receive HBOT within the first 24 hours of ICU admission (because they are deemed too unstable for HBOT) vs. those who receive HBOT within the first 12 and 24 hours of ICU admission

Study III

Study III hypotheses

In NSTI patients stratified into no sepsis, sepsis, and septic shock groups as defined by standardized criteria (45), suPAR levels are a predictor for mortality.

In NSTI patients stratified into no sepsis, sepsis, and septic shock groups as defined by standardized criteria (45), suPAR levels reflect NSTI patients' clinical condition as assessed by

Simplified Acute Physiology Score II (SAPS II) and Sequential Organ Failure Assessment (SOFA) scores.

Study III primary endpoint

Association between plasma suPAR levels, measured upon admission and once daily during the first three days in the ICU, and NSTI mortality, SAPS II and SOFA scores.

Joint secondary endpoints for studies I, II and III

In study I, II and III, the following outcomes will be analyzed for the NSTI group only:

- Mortality in the ICU and at 30, 90 and 180 days
- Amputations

The following characteristics are registered in the INFECT database, which we will also be using for our studies:

- Age and sex
- Comorbidities: diabetes mellitus, liver cirrhosis, kidney disease, cardiovascular disease, HIV/AIDS, hepatitis, intravenous drug use, malignancy
- Body mass index (BMI)
- Mean arterial pressure (MAP)
- Heart rate
- Arterial blood gas: pO_2, pCO_2, HCO_3, base excess, pH
- Standard biochemistry: K^+, Na^+, Ca2+, glucose, creatinine, haemoglobin etc.
- Norepinephrine use
- Mechanical ventilation
- ICU scores: SAPS II, SOFA without GCS, LRINEC
- Primary infectious focus
- Primary symptoms: pain, erythema, tachycardia, fever
- Pathogen type
- Time between admittance at primary hospital to the first surgery
- Definitive treatment at Copenhagen University Hospital: antibiotics, immunoglobuline and HBOT
- Treatment at primary hospital: antibiotics, immunoglobuline, surgical treatment

- Immunocompromising drugs prior to admission

4.6 Sample size

Study I

The test kits we will be using to measure our primary outcome sTM (Human sCD141 ELISA kit, *Nordic Biosite*) have an interassay standard variation of 0.58 ng/ml. In order to be certain that measured changes in sTM concentration are not a result of interassay standard deviation, we have set our mimimum relevant difference in sTM to 3 x the interassay standard variation, thus 1.75 ng/ml.

We prepared a power calculation using a Wilcoxon rank sum test. Assuming an estimated standard deviation of 4.6 ng/ml and a mean of 9.9 ng/ml (12), we will need to include a maximum of 150 NSTI patients and 50 elective surgery patients to reach a statistical power of at the very least 60 % (a very conservative estimate) and presumably closer to 85 % (more realistic estimate) at a 5 % significance level. The estimates depend on data distribution.

Study II

The test kits we will be using to measure our primary outcome sE-selectin (Human CD62E ELISA kit, *Diaclone*) have an interassay standard variation of 0.37 ng/ml. In order to be certain that measured changes in sE-selectin concentration are not a result of interassay standard variation, we have set our mimimum relevant difference in sE-selectin to 3 x the interassay standard variation, thus 1.1 ng/ml.

Assuming an estimated standard deviation of 209 ng/ml (septic shock) vs. 23 ng/ml (severe sepsis and sepsis) and means of 295 vs. 181 ng/ml, respectively (46), we will need to include at least 132 NSTI patients and 50 elective surgery patients to reach a statistical power of 90 % at a 5 % significance level.

Study III

suPAR levels during NSTI have never previously been examined. In order to estimate sample size and since most NSTI patients are also septic, we are basing our sample size calculation on a previous study concerning the correlation between suPAR and sepsis (29). This study found statistically significant correlation between suPAR levels and mortality in 141 patients. This is also

our goal. Further studies have also found significant correlations between suPAR, sepsis and mortality in 132 patients (30). We will include at least 150 NSTI patients during this study.

4.7 Statistical considerations

Studies I and II

To check whether the HBOT treatment has an effect on the range of biomarkers, we will analyze the means and variances of the biomarkers in the NSTI group and the two control groups, the orthopaedic patients and the sepsis patients. Non-parametric data will be log-transformed and will be presented as median values with IQR. Wilcoxon rank sum tests will be used for group comparisons. Fisher's exact test will be used for categorical data. Correlation analysis will be performed using Spearman rank correlation or Pearson correlation.

Study III

To assess the quality of suPAR as a predictor of health outcomes, a model selection exercise will be conducted with various types of regression models. The type of regression will vary with the type of health-outcome, with suPAR as the predictor in all cases. Non-parametric data will be log-transformed and will be presented as median values with IQR. Fisher's exact test will be used for categorical data. Receiver operating characteristic (ROC) curve analysis will be applied to determine suPARs accuracy as a marker of severity and mortality in patients with NSTI. We will construct Kaplan-Meier curves for survival data. Statistically significant results are when $p<0.05$. Corrections for multiple comparisons will be done using Wilcoxon rank sum (HDR) tests.

4.8 Ethics and dissemination

The study will be conducted in accordance with the principles of the Declaration of Helsinki. The Regional Health Research Ethics Committee and the Danish Data Protection Agency (responsible for the correct processing of confidential patient data) have approved the study (RHREC doc. nr.: H-16021845; DDPA j.nr.: RH-2016- 199). The investigator will inform the Research Ethics Committee and the Danish Data Protection Agency of any significant changes to the protocol.

Written informed consent will be acquired from either the patients themselves or their next of kin as well as from their primary healthcare physician, as required by Danish law. This study itself poses no additional risk to the patients, as patients will receive standard NSTI treatment at Copenhagen University Hospital, in no way different from the usual treatment. To maintain

confidentiality, each patient is assigned a pseudonymous research code. Access to patient data analysis is restricted to the investigators.

The study has been registered at the international database of clinical trials at www.clinicaltrials.gov NCT03147352.

Results will be disseminated at national and international conferences and then published in international peer-reviewed scientific journals. Positive, negative and any inconclusive results will be published. The advanced knowledge of NSTIs generated by the above studies will be used to create evidence based guidelines for classification and management. Through the INFECT project, we have access to the UK NSTI patient organization together with the NSTI clinical consortium. This provides excellent means for efficient dissemination of guidelines and other advances made in the project to relevant end-users, including medical staff, patients and their relatives, small and medium enterprises (SMEs) and researchers.

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4.9 Authors' contributions

PP and OH designed and wrote the research protocol. PP is responsible for the sample size calculations and statistical methods. PP is responsible for data acquisition. PJ contributed to drafting the protocol. PJ is responsible for the laboratory work in study I and II.

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4.11 Competing interests

None declared.