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Use of oxygen-ozone therapy to improve the effectiveness of antibiotic treatment on infected arthroplasty: protocol for a superiority, open-label, multicentre, randomised, parallel trial

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ABSTRACT

Introduction Surgical site infections still remain a major public health challenge and have become an increasing universal risk, especially for the implantation of orthopaedic devices.

Unfortunately, the discovery and increasingly widespread use (especially the misuse) of antibiotics have led to the rapid appearance of antibiotic-resistant strains today; more and more infections are caused by microorganisms that fail to respond to conventional treatments.

Oxygen-ozone therapy has been extensively used and studied for decades across various potential medical applications and has provided consistent effects with minimal side effects.

This study aims to determine the superiority of oxygen-ozone therapy in combination with oral antibiotic therapy in patients with wound infections after an orthopaedic device implantation when compared with antibiotic therapy alone.

Methods and analysis This is an open-label, multicentre, randomised, parallel-group study that aims to assess the efficacy and safety of oxygen-ozone therapy in combination with oral antibiotic therapy to treat infections in patients (male or female aged ≥18 years) having undergone surgery for the implant of an orthopaedic device. Patients must have at least one (but no more than three) postoperative wounds in the site of surgery (ulcers, eschars and sores) and at least one symptom (pain, burning, redness and malodour) and at least one sign (erythema, local warmth, swelling and purulent secretion) of infection of at least moderate intensity (score ≥2) in the target lesion at the screening visit (patients with wounds without signs of localised infection or with undermining wounds will be excluded).

Patients (n=186) will be recruited from five Italian hospitals and studied for 7 weeks. All will be assigned to one of the two treatment groups according to a web-based, centralised randomisation procedure and placed into either the (1) intervention: oxygen-ozone therapy 2–3 times a week for 6 weeks (for a maximum of 15 sessions) simultaneously with an appropriate oral antibiotic therapy prescribed at baseline or (2) control: oral antibiotic therapy prescribed at baseline.

The primary outcome is the efficacy and superiority of the treatment (ozone and oral antibiotic therapies); secondary outcomes include the resolution of signs and symptoms, modifications in lesion size and the treatment's safety and tolerability.

Ethics and dissemination This study has been reviewed and approved by the responsible Independent Ethics Committee (IEC) of COMITATO ETICO CAMPANIA NORD, located at 'Azienda Ospedaliera San Giuseppe Moscati di Avellino'.

After completion of the study, the project coordinator will prepare a draft manuscript containing the final results of the study on the basis of the statistical analysis.

The manuscript will be derived by the co-authors for comments, and after revision, it will be sent to a major scientific journal. Findings will be disseminated via online and print media, events and peer-reviewed journals.

Trial registration number NCT04787575.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This is the first trial assessing the superiority of oxygen-ozone therapy in combination with antibiotic treatment for the treatment of infections following the implantation of orthopaedic prostheses.
⇒ Patients are expected to adhere to the trial easily as the intervention will be performed during a hospital stay.
⇒ The study will be conducted in Italy so most participants will be Italian and conclusions cannot be fully extrapolated to general populations worldwide.
⇒ This study design involves an open-label trial, as it is needed both for participants and care providers to be aware of it for the proper administration of the intervention.

INTRODUCTION

Disease background

The use of orthopaedic devices has revolutionised the treatment of patients with debilitating diseases like osteoarthritis and bone fractures.[1] However, when used, these may

predispose the body to infection and make orthopaedic surgery a challenge.

Patients who suffer a surgical site infection experience a longer hospital stay in addition to higher morbidity–mortality rates and hospital costs.[2] Considering the risks and mechanisms of infection development following the implantation of orthopaedic devices, especially in the case of infections related to biofilm formation,[3] an expected increase in the number of surgically placed orthopaedic implants in the coming decades, as well as a need for increased attention to antibiotic resistance, there is a clear necessity for improved technologies to prevent, diagnose or treat orthopaedic device-related infections.

Therefore, research efforts have been initiated in this area, with numerous clinical studies having reported orthopaedic device-associated infections and clinical studies focusing on antibiotic resistance and its prevalence in orthopaedic implants.[4]

Other researchers have also identified associations between factors such as device size, anatomical location and comorbidities as causes for the variability of infection rates for device classes.[5] Finally, other factors, such as pathogen species, have also demonstrated an effect on the infection timing of early onset or delayed infections.[5]

Investigational product

Ozone is a colourless gas made up of three oxygen molecules; it is a natural part of the environment, together with oxygen.

In recent years, ozone has shown many useful roles in medical therapy. For example, inactivating Legionella in contaminated water, controlling pain, infections and inflammation in diabetic, epithelial, surgical and complicated wound healing, and treating ulcers without serious adverse effects. These treatments may be even more widely known as integrative medicine, considering their efficiency and safety.[6–8]

Medical ozone gas is created using a corona high arc discharge.[6] It has been used for decades and has proved to be effective and consistent, with minimal side effects.[7] Ozone therapy works by stimulating oxygen metabolism, increasing the production of red blood cells and improving the rheological properties of the blood.[8] It also induces the production of prostacyclin, a potent vasodilator.[9] Other proven effects of ozone have been its ability to promote positive immunological reactions in the body[10] and its bactericidal action.[11]

Rationale

The evidence regarding orthopaedic device-associated infections, their prevalence and their related antibiotic resistance is constantly growing.[4] While the number of methicillin-resistant *Staphylococcus aureus* (MRSA) cases is rapidly growing globally, the most frequent causes of infections with antibiotic-resistant bacteria in orthopaedic devices remain those caused by *Staphylococcus* (*S. aureus* and *S. epidermidis* predominantly). Specifically, *S. aureus* is the first cause in terms of prevalence for orthopaedic device-associated infections with both methicillin-susceptible and methicillin-resistant strains.

It has been clearly demonstrated that infections with antibiotic-resistant bacteria have a poorer outcome than those susceptible to antibiotics.[4]

Overall, ozone treatment has proven to be highly beneficial to patients with infectious diseases and has led to rapid healing and accelerated cicatrisation.[12] In humans, ozone has been effectively used as an antibacterial agent to treat oral infections caused by *Actinomyces naeslundii*, *Lactobacilli casei* and *Streptococcus mutans*.[13] In a study conducted in 86 patients with chronic wounds, oxygen-ozone therapy was associated with significant improvements in the healing of chronic wounds.[14] In another study, 200 patients with diabetic foot ulcers were randomised to receive oxygen-ozone therapy plus routine treatment for diabetic foot care and all patients in the ozone group completed wound closure.[15]

Additionally, evidence from millions of patients over the last 40 years has proven oxygen-ozone therapy to be exceptionally safe,[16] with a complication rate of 0.7 per 100 000 treatments, usually attributable to improper administration.[6]

Based on this background, the research hypothesis of this study is to investigate whether the use of oxygen-ozone therapy given in combination with oral antibiotic therapy may produce further benefits than oral antibiotic therapy alone for the treatment of infections following the implant of orthopaedic prostheses. Furthermore, this study will allow us to evaluate the efficacy of treatment with oxygen-ozone therapy (Scientific Society of Oxygen Ozone Therapy (SIOOT)) plus oral antibiotic therapy on bacteria strains known to be associated with antibiotic resistance, that is, recovery of efficacy versus these bacterial strains.

SIOOT is a scientific association that aims to promote research and studies for the development and application of oxygen-ozone therapy.

Treatments are provided through the Grande Auto-Emoinfusione, a haemoinfusion machine that administers a daily dose of ozone therapy (for a maximum of two times per day) for the 6–15 days following the start of the signs and symptoms of infection.

Summary information on this trial according to the WHO Trial Registration Data Set is available in table 1.

Primary and secondary endpoints

The primary objective of this trial is to evaluate the efficacy of treatment with oxygen-ozone therapy plus oral antibiotic therapy in comparison to oral antibiotic therapy alone in patients with infections following the implant of orthopaedic prostheses.

Among its secondary objectives, this trial aims to:

- Evaluate the efficacy of treatment on
  1. Resolution or improvement of signs and symptoms (ie, a score ≤1 for a maximum of two signs or symptoms) on Day 7, Day 14 and Day 28 and after treatment ends. The following symptoms of

Table 1 Study steps and timeline

Visit number	V_1 Screening	V_2 baseline/start of treatment	V_3	V_4	V_5	V_6 final visit/end of study
Time (days)	−7/−3	0	7±2	14±2	28±2	42±3
Written informed consent	X					
Demographic data	X					
Vital signs (1)	X	X	X	X	X	X
Measurement of body temperature	X	X	X	X	X	X
Physical examination	X					
Medical history and concomitant diseases	X					
Prior and concomitant medication (2)	X	X	X	X	X	X
Urine pregnancy test (3)	X					
Identification and evaluation of the target lesion	X(4)					
Photo of the target lesion	X		X	X	X	X
Measurement of the area of the target lesion	X		X	X	X	X
Evaluation of the clinical signs of infection	X	X	X	X	X	X
Cleaning or medication of lesion(s)	X	X	X	X	X	X
Swab collection of the infected target lesion	X(5)			X		X
Study eligibility (inclusion and exclusion criteria)	X	X				
Randomisation		X				
Schedule of oxygen-ozone therapy (6)		X	X	X	X	
Prescription of oral antibiotic therapy (7)		X		X		
Bacteriological response				X		X
Investigator's global assessment				X		X
Laboratory parameters	X			X		X
Assessment of compliance to antibiotics			X	X	X	X
Adverse events	X	X	X	X	X	X

infection will be evaluated by patients on a Likert scale of 0–4 (0 = no symptom; 1 = mild symptom; 2 = moderate symptom; 3 = severe symptom; 4 = very severe symptom): pain, burning, redness and malodour. The following signs of infection will be evaluated by investigators on a Likert scale of 0–4 (0 = no sign; 1 = mild sign; 2 = moderate sign; 3 = severe sign and 4 = very severe sign): erythema, local warmth, swelling and purulent secretion.

2. Bacteriological outcome (eradication of the pathogen isolated at the screening visit without superinfection or reinfection with the same pathogen) on Day 14 and Day 28 and at the end of treatment. The following definitions will be assigned by the investigator in the assessment of bacteriological response at Visit 4 (Day 14), based on the analysis of the swab collected at the screening visit (V1) (in the target lesion and in the case of multiple lesions).
   - Eradication: elimination of a pathogen identified at the screening visit (V1).
   - Persistence: presence of the pathogen identified at the screening visit (V1).
   - Superinfection: identification of a pathogen not identified at the screening visit (V1).

   The following definitions will be assigned by the investigator in the assessment of bacteriological response at Visit 6 (Day 42), based on the analysis

of the swab collected in the previous visits (in the case of the target lesion and in the case of multiple lesions):
  - Eradication: elimination of a pathogen identified at the screening visit (V1) and/or in a previous visit. Full eradication of all pathogens is needed to satisfy this definition.
  - Persistence: presence of the pathogen identified at the screening visit (V1) and/or in a previous visit.
  - Superinfection: identification of a pathogen not identified at the screening visit (V1) and/or in a previous visit.
  - Reinfection: the reappearance of a pathogen identified at the screening visit (V1) and eradicated in a previous visit.
3. Changes in lesion size (measurement of the area of the target lesion, which will be carried out using a digital camera) from baseline to the end of the 14 days of treatment and at 28 days after the end of the treatment.
4. Changes from baseline to any postbaseline time point in body temperature.
5. Improvement of laboratory parameters (laboratory safety parameters, haematology and blood chemistry will be evaluated in the reference local laboratory of each investigational study site during the screening visit (V1), Day -7/-3; at Visit 4, Day 14 and at Visit 6, Day 42). The following laboratory parameters will be measured:
  - Haematology: haemoglobin, haematocrit, red blood cell count, erythrosedimentation rate, white blood cell count with differential count and platelet count;
  - Blood chemistry: high-specificity C reactive protein, creatinine, blood urea nitrogen, glucose, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase, total bilirubin and electrolytes (sodium, potassium, magnesium, chloride and calcium).

  Furthermore, safety laboratory tests may be performed at any other time during the study, if deemed necessary by the investigator.

  All female patients of childbearing potential will perform a serum pregnancy test at the screening visit (Visit 1, Day −7/−3).

- Evaluate the safety and local tolerability of treatment with oxygen-ozone therapy in comparison with antibiotic therapy alone.

The trial is an open-label, multicentre, randomised, parallel-group study with a 1:1 allocation ratio consisting of 186 evaluable patients whose purpose is to evaluate the superiority of oxygen-ozone therapy in addition to oral antibiotic treatment compared with antibiotic therapy alone.

METHODOLOGY

Study setting

The study will be performed at five public hospitals in Italy (see online supplemental material), and it will start with the patient enrollment phase that will last 20 months (from January 2024 to September 2025). The study will end by January 2026 to allow the patients enrolled in September 2025 to complete their 4 months of follow-up.

This is a superiority study designed to evaluate the efficacy and safety of oxygen-ozone therapy plus oral antibiotic therapy in the treatment of infections due to orthopaedic prostheses.

The sample size is discussed for the primary efficacy endpoint, defined as the proportion of patients with clinical success on Day 14.

The following assumptions are made for the calculations:

- Proportion of patients having clinical success on Day 14 in the oral antibiotic therapy alone group: 50%.
- Proportion of patients having clinical success on Day 14 in oxygen-ozone therapy plus oral antibiotic therapy: 70%.
- Difference between the group proportions: 20%.

With a power of 80% and a significance level of 0.05 (two-sided), 93 patients in each treatment group are required. The test statistic used is the two-sided Z-test with pooled variance.

Considering a rate of dropouts of up to 10%, up to 104 patients in each treatment group may be enrolled.

Eligibility criteria: inclusion criteria

To be included in the study, patients must:

- Be aged >18 years old.
- Have received an orthopaedic prosthetic implant surgery in the previous 8 weeks.
- Present 1–3 postoperative, non-overlapping wounds on the surgery site, with a target wound area of <100 cm^2 (in case of multiple wounds, however, not more than three non-target lesions must not overlap with the target one, that is, the largest one).
- Show at least one symptom and one sign of infection of moderate or higher intensity (signs and symptoms will be evaluated by patients on a Likert scale of 0–4 points (0=no symptom; 1=mild symptom; 2=moderate symptom; 3=severe symptom and 4=very severe symptom) in the target lesion that are confirmed at their baseline visit.
- Be pathogen-positive at the wound swab test (patient with the presence of at least one pathogen identified in the swab collection in the target lesion that is amenable to being eradicated with oral antibiotic therapy).
- Patients should be able to receive or perform self-care at home and be willing to refrain from all non-permitted concomitant medication (topical antibiotics or parenteral antibiotics or any other antibiotics in addition to the prescribed systemic oral antibiotic therapy; corticosteroids by any route; chemotherapeutic agents or radiation therapy or

immunosuppressive therapy; silver-based or hyaluronic acid-based products; any hydrating and/or moisturising cream and bioactive dressings delivering antimicrobial substances or other bioactive components) during the entire study.
- Obtain a negative pregnancy test in the case of women of childbearing age and agree to take appropriate precautions to delay pregnancy through the study (the study entry criteria require that childbearing potential women use appropriate contraceptive methods, and participants with positive pregnancy tests are excluded from the study). If a patient becomes pregnant during the course of the study, the investigational product must be discontinued immediately.
- Be able to read, understand, comply with procedures and sign an informed consent form (ICF).

Eligibility criteria: non-inclusion criteria

Patients are not eligible for trial enrollment if any of the following criteria are satisfied:
- Wounds don't have signs of localised infection (ie, pain, erythema, oedema, heat, purulent exudate, serous exudate with concurrent inflammation, delayed healing, discolouration of granulation tissue, friable granulation tissue, pocketing at the base of the wound, foul odour and wound breakdown).
- There are more than four wounds, or any wound is >100 cm^2.
- There is a presence of undermining wounds.
- Patient has favism (ie, deficiency of the glucose-6-phosphate dehydrogenase enzyme), uncontrolled hyperthyroidism (an untreated condition in which the thyroid gland makes too much thyroid hormone) or a history of connective tissue disease.
- An active malignant disease (a cancerous tumour) is present.
- Patient is a candidate for any surgery during the study duration.
- Usage of topical corticosteroids by any route in the previous 4 weeks or systemic corticosteroids in the previous 7 days.
- Treatment with hydrating or moisturising cream within 24 hours or less of the study.
- Patient is receiving chemotherapy, radiation or immunosuppressive therapies.
- Patient has contraindications to antibiotic therapy.
- Pregnant or breastfeeding women or patients with childbearing potential who are not taking appropriate precautions to delay pregnancy during the study.
- Participation in any other clinical research 30 days or less prior to consenting to the study.
- An inability to understand informed consent and a high probability of non-compliance with the study according to the investigator's judgement, including patients with other major diseases deemed clinically significant by the investigator or which, according to his or her professional knowledge, would interfere with the study.

Treatment

At the baseline visit (Visit 2, Day 0), eligible patients will be randomised to one of the two following treatment groups:
- Oxygen-ozone therapy SIOOT plus antibiotic therapy.
- Antibiotic therapy.

Administration method

Oxygen-ozone therapy

The oxygen-ozone therapy will be provided using MEDICAL 95 CPS, an ambulatory unit for oxygen-ozone therapy, which customises the gaseous mixture according to clinical needs. It is a certified equipment (medical device as per Directive 93/42/EEC and S.M.I. in 2A class) produced by MULTIOSSIGEN SPA (Bergamo, Italy), complying with SIOOT protocols.

It will be performed by:
- Self-haemoinfusion of 200 cc with concentrations of 40–50 µg/mL, to be performed 2–3 times a week, for a time of 6 weeks (for a maximum of 15 sessions). A volume of 200 cc will be drawn from an antecubital vein and placed in a certified bag. Without detaching the patient from the needle used to take the blood sample, a proper device enters a gas mix of ozone and oxygen in the bag and mixes it with the drawn blood. Once the tourniquet is removed from the patient's arm, the bag is lifted, and the blood loaded with ozone and oxygen refluxes into the patient's body.
- Subcutaneous injections in the perilesional site at a dose of 5 cc with concentrations of 4 µg/mL.
- Cleanse wounds with 100 cc of 5–10 ug of ozone gas.

Oral antibiotic therapy

In both treatment groups (group A and group B), appropriate oral antibiotic therapy will be prescribed at baseline and (in cases of persistency, superinfection or reinfection) at follow-up visits. The choice of antibiotic will be at the discretion of the investigator, based on the results of the culture of the swab collected in the target lesion at the screening visit (and later, if needed) and the associated antibiogram. The dose and duration of treatment with antibiotic therapy will be based on clinical and bacteriological requirements, as well as on information on the prescription for each antibiotic drug (as per the clinical practice).

The designated site staff of each investigational study site is responsible for the prescribed oral antibiotic accountability, reconciliation and record maintenance as per local standard clinical practice and in accordance with local regulatory requirements.

No assessments of compliance to treatment with oxygen-ozone therapy SIOOT (group A) are scheduled, as oxygen-ozone therapy SIOOT will be administered by experienced operators, who will ensure the correctness of the procedure.

Patients will be instructed to keep the unused units of prescribed oral antibiotics in order to allow the investigator to evaluate the patient's compliance with treatment

at the first visit after the end of treatment with antibiotics. The evaluation of compliance with prescribed oral antibiotics will be done in the safety population by using the following formula:

$$\frac{\text{TOTAL NUMBER OF ADMINISTERED DOSS}}{\text{TOTAL NUMBER OF SCHEDULED DOSES}} \times 100 = \%\ \text{OF ADMINISTERED DOSES}$$

The total number of administered doses will be calculated by subtracting the number of unused doses returned at the first visit after the end of treatment with antibiotics from the number of doses prescribed at the baseline visit (or later, if needed).

The compliance to treatment with the prescribed oral antibiotics will be categorised as excellent (90%–110% of scheduled), good (75%–89% or 111%–125% of scheduled), poor (25%–74% or 156%–175% of scheduled) or none (175% of scheduled).

Outcomes

Primary outcome

The proportion of patients with clinical success on Day 14 (Visit 4), defined as resolution or improvement of signs and symptoms of infection of the wound in the target lesion (ie, a score ≤1 for a maximum of two signs or symptoms) from baseline to Day 14. Investigators will be requested to score the outcome of the target lesion on a five-grade scale: 1=worsening, 2=no change, 3=minimal improvement, 4=moderate improvement and 5=good improvement or resolution.

Secondary outcomes

The proportion of patients with clinical success (resolution or improvement of signs and symptoms of infection of the wound in the target lesion) on Day 7 (Visit 3), Day 28 (Visit 5) and Day 42 (Visit 6):

- Changes to the score in total or individual signs and symptoms from baseline to any postbaseline checkpoint.
- Changes in body temperature and/or target lesion size from baseline to any postbaseline checkpoint.
- Length of time for resolution of all signs and symptoms of infection in the target lesion.
- Bacteriological success (eradication of the pathogen isolated at the screening visit without superinfection or reinfection with the same pathogen) of the wound in the target lesion on Day 14 and Day 42.
- Investigators' global assessment (investigators will be requested to score the outcome of the target lesion on a five-grade scale: 1=worsening, 2=no change, 3=minimal improvement, 4=moderate improvement and 5=good improvement or resolution) of the target lesion on Day 14 and Day 42.
- Changes of laboratory parameters indicative of infection from baseline to Day 14 and Day 42.

The results of changes from baseline to any postbaseline time point of total symptoms score and score of individual signs and symptoms, changes from baseline at the end of the 14 days of treatment and at 28 days after the end of treatment of the size of the target lesion, changes from baseline to any postbaseline time point in body temperature and changes from baseline at the end of the 14 days of treatment and to 28 days after the end of treatment of laboratory parameters indicative of infection will be analysed by means of an analysis of covariance (ANCOVA) for repeated measures (Proc GLM). The change from baseline over the study period will be the dependent variable, while treatment group, visit and treatment group by visit interaction will be considered as fixed factors of the model, while the baseline values will be considered as covariates of the model. The adjusted means for both treatment groups at each time point with 95% CIs and p values will be presented. The difference between the adjusted means for the two treatment groups at each time point and overall will be calculated with the 95% CI. The results of the time to resolution of all signs and symptoms of infection will be analysed using a log-rank model, and Kaplan-Meier estimates will be computed.

Patient and public involvement

Patients who previously had wounds related to orthopaedic transplants were not directly involved in the initial research, research questions, outcome measures, study design, recruitment phase or burden of interventions. However, previous patients' experiences were taken into consideration for the preparation of all these aspects.

The intent is to disseminate the main results to trial participants and to seek patient and public involvement in the development of an appropriate method of dissemination.

Participant timeline

The total study duration will be 14 months, with patients being enrolled in the study for approximately 12 months and each patient receiving intervention for 7 weeks, including a run-in period and treatment phase. During the screening period, eligible patients will enter a 3–7 run-in to determine their inclusion and exclusion criteria and receive a clinical evaluation. After patients are selected for the study, Visit 2 will include the baseline visit for the randomisation process to start as well as treatment with oxygen-ozone therapy (group A) and oral antibiotic therapy (both groups). The period of treatment with the oxygen-ozone therapy for group A will last 6 weeks. The period of treatment with oral antibiotic therapy will be at the discretion of the investigator (table 1).

Sample size

The study will include a total of 186 evaluable patients, 93 in each treatment group. Considering a dropout rate of 10%, up to 104 patients in each group will be enrolled. Being a superiority design, the sample size is discussed for the primary efficacy endpoint, defined as the proportion of patients with clinical success on Day 14.

For the calculations, it has been assumed that:

1. Ffity per cent of patients receiving antibiotic therapy alone and 70% of patients receiving antibiotic

oxygen-ozone therapy will experience clinical success, with a difference between group proportions of 20%.
2. With a power of 80% and a significance level of 0.05 (two-sided), 93 patients in each treatment group are required.
3. The statistical test used is the two-sided Z-test with pooled variance.

Recruitment

Five location sites across Italy have been selected to maximise the probability of recruiting a sample sufficient for the trial (n=186), as well as to align their characteristics with the inclusion criteria in the study. Sites with a high number of implantations of orthopaedic prostheses are involved in trials; so investigators will select evaluable patients to be subjects if they have undergone surgery for an implant of an orthopaedic prosthesis in the previous 8 weeks, have at least one wound in the site of surgery and have at least one sign and one symptom of infection of at least moderate intensity.

Randomisation

The randomisation list will be in a balanced-block design and will be prepared using a validated system that automates the random assignment of treatment groups to randomisation numbers.

The investigator will assign a screening number (two-digit site number and two-digit progressive number for each site) to each patient included in the study. Patients will be sequentially assigned to the next screening number as they present themselves for the study at the screening visit (Visit 1), starting from number 1.

On verification of all the inclusion and exclusion criteria, eligible patients will be assigned to the next lowest randomisation number (three-digit randomisation number) available, based on the number of available kits on site.

If a subject discontinues from the study, both the screening and the randomisation number will not be reused, and the subject will not be allowed to re-enter the study.

For each treatment (ie, for each number of randomisation), the investigator will receive a sealed envelope containing the code of the product taken by the patient.

Assignment of interventions

Allocation

No stratification procedures are planned for the treatment group assignment in this study. No predetermined subgroups of patients are defined in the study. If considered appropriate, subgroup analysis by variables that are considered relevant may be defined in the final statistical analysis plan.

Blinding or masking

The nature of the intervention (the oxygen-ozone therapy) made blinding impossible and unfeasible. In order to control potential biases, we decided to randomly assign participants to the different treatment groups. We also decided to collect comprehensive baseline data on participants' characteristics and adjust for any imbalances during statistical analysis. We finally decided to adhere to the reporting guidelines, Consolidated Standards of Reporting Trials, to ensure transparent and complete reporting of trial results.

DATA COLLECTION, MANAGEMENT, ANALYSIS AND SHARING PLAN

Data collection methods

This study will use an electronic data capture (EDC) system, an eClinical platform provided by Clinical Research Technology. This is fully validated, secure, web-enabled software that conforms to Food and Drug Administration requirements. The designated investigator staff will enter the data required by the protocol into the electronic case report form (eCRF). Investigator site staff will not be given access to the EDC system until they have been properly trained.[17]

Automatic validation programmes will check for data discrepancies in the eCRFs and allow modification or verification of the entered data by the investigator staff. As it is understood that an excessive rate of withdrawals can render the study uninterpretable, efforts will be made to promote participants' retention in the study and avoid unnecessary withdrawals. The strategies to promote participants' retention include a clear informed consent process and the establishment of a trusted relationship based on trust and engagement.

However, the study has been designed to function properly even in the case of a 10% withdrawal rate. In the case of patients deciding to discontinue treatment prematurely, contact will be maintained if the patient agrees to provide further information. In the case of premature withdrawal, the same assessment described for Visit 6 (Day 42±3) will be performed and recorded in an 'Early termination visit'. If the reason for the removal of a patient from the study is an adverse event (AE), the patient should be followed until its resolution.

Data management

The investigational staff involved in the study will be adequately trained by representatives of the Contract Research Organisation (CRO) before any activities related to the study are initiated. The sponsor personnel (or designated CRO) will review the data entered by investigational staff for completeness and accuracy, and the occurrence of any protocol violations will be determined. After the data have been verified to be complete and accurate, the database will be declared locked.

Statistical methods

The descriptive statistics will be provided in summary tables by treatment group according to the type of variable and demographics, and baseline characteristics data will be summarised by means of descriptive statistics as well.

For the comparison between the two treatment groups on the primary efficacy endpoints, a χ^2 test will be used. In addition, a sensitive analysis will be performed to reference the size of the target lesion at baseline using a logistic regression model.

Any changes from baseline to any postbaseline checkpoint of a patient's total symptoms score, the score of individual signs and symptoms, as well as other changes in size, body temperature and laboratory parameters, will be analysed by means of an ANCOVA for repeated measures (Proc GLM).

All treatment-emergent AE (TEAE) will be prepared, showing a number of TEAEs together with the number and percentage of patients with any TEAEs, treatment-related TEAEs, serious TEAEs, treatment-related serious TEAEs, severity of TEAEs and TEAEs leading to withdrawal. The results of vital signs and safety laboratory parameters will be summarised by treatment group and visit by means of default descriptive statistics.

DATA MONITORING

A data monitoring committee (DMC) was not applicable to this study. The study duration per subject represented a short window of time at just 7 weeks, including the post-treatment follow-up period, and the drugs under investigation are well known. These reasons are presented as valid for not setting up a DMC according to the European Medicines Agency guidelines on DMCs (Ref. EMEA/CHMP/EWP/5872/03). No interim analyses, data monitoring or stopping rules before the conclusion of the study are planned.

AEs, past and concomitant diseases will be monitored and coded using the Medical Dictionary for Regulatory Activities (MedDRA). In the case of any serious adverse event (SAE) taking place during the study and after the investigator determines that the event(s) meet the protocol definition of an SAE, it will be reported immediately (ie, within 24 hours) to the Sponsor Drug Safety Officer, regardless of whether or not it is related to the investigational product. SAEs will be followed up until resolution, stabilisation or the patient is lost to follow-up.

TEAEs will be tabulated by primary System Organ Class and Preferred Term after medical coding using the MedDRA. TEAEs with a 'definite', 'probable' or 'possible' relationship to the investigational product will be considered as treatment related; missing data concerning the investigational product's relationship will also be considered as treatment related.

If a patient dies during participation in the study or during a recognised follow-up period, the investigator should send any other available postmortem information, including autopsy and histopathology as well as the SAE form to the sponsor.

Source data and documents will be available for inspection by the sponsor or health authorities. Representatives of the sponsor may visit a participating site at any time during or after the completion of the study to conduct an audit.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics approval

This protocol and the proposed Informed Consent Form (ICF) are approved by the responsible Independent Ethics Committee (IEC) of COMITATO ETICO CAMPANIA NORD located at 'Azienda Ospedaliera San Giuseppe Moscati di Avellino'. Additionally, before the start of data collection, the participant's physician (or the study sponsor or CRO) will provide the IEC with a current and complete copy of the following documents:

- Final protocol and, if applicable, protocol amendments.
- Sponsor-approved participation agreement/ICF (and any other written materials to be provided to the patients).
- Participating physician 's curriculum vitae or equivalent information (unless not required, as documented by the IEC).
- Information regarding the name of the sponsor, institutional affiliations, and potential conflicts of interest.
- Any other documents that the IEC requests to fulfil its obligations.

All the procedures were followed in accordance with the relevant guidelines.

All experimental protocols were approved by a named institutional and/or licensing committee ('COMITATO ETICO CAMPANIA NORD' Independent Ethics Committee).

The IEC has given full approval of the final protocol, protocol amendments and the participation agreement/ICF, and the sponsor has received a copy of this approval. At the end of the study, where required by local regulations, the participating physician (or sponsor, where required) will notify the IEC about the study's completion.

Neither the participating physician nor the sponsor can modify this protocol without a formal amendment by the sponsor. Protocol amendments would not be implemented without prior IEC approval, where applicable.

Consent to participate

Informed consent was obtained from all subjects and/or their legal guardian(s).

At the screening visit, the investigator will inform the patient about the clinical study and all associated procedures. The information sheet will be given to the patient who will have time to read it and ask questions about the study. The patient will decide freely whether to participate or not. If he/she decides to participate, he/she will be asked to sign the ICF. The original signed ICF will be retained in the investigator's site file and a copy will be provided to the participant. Individuals will be free to decline further participation without giving reasons.

Consent for publication

Informed consent was obtained from all subjects and/or their legal guardian(s).

Ancillary studies are not planned for this trial, but in the case ancillary studies are willing to be performed using the collected data, a signed consent from every participant of the ancillary study will be obtained first.

Availability of data and materials

Data collection

Information about the study's subjects will be kept confidential and managed under the applicable laws and regulations. The data collection system for this study uses built-in security features to encrypt all data for transmission in both directions, preventing unauthorised access to confidential participant information. Access to the system will be controlled by a sequence of individually assigned user identification codes and passwords, made available only to authorised personnel who have completed prerequisite training.

The investigator will ensure the anonymity of the patients. Patients will not be identified by names in any documents submitted to the sponsor. The sponsor will maintain confidentiality standards by giving each patient enrolled in the study a unique patient identification number.

The data that support the findings of this study are available from the principal investigator of the study, Dr Fidelia Cascini (fidelia.cascini1@unicatt.it), but restrictions apply to the availability of these data, which were used under licence for the current study and so are not publicly available. Data are, however, available from the principal investigator on reasonable request and with permission of the ICF (or separate authorisation for use and disclosure of personal health information) signed by the patient, unless permitted or required by law. Data generated by this study will be available for inspection on request by representatives of national and local health authorities, marketing authorisation holder monitors, representatives, collaborators and the IEC for each study site, as appropriate.

Patient's coverage and ancillary studies

The study promoter has undersigned an insurance policy covering subjects who enter the study. References are included in the information sheet for the subject. The study promoter will also indemnify the investigator for damages outside of any act of omission on his or her part or those under his or her supervision that shall or may amount to negligence in law. No ancillary studies are intended for this trial.

Dissemination

According to the ICH Guidelines on Good Clinical Practice, the sponsor of a study is the owner of the data resulting from the study. All centres and investigators participating in the study should not disseminate information or data without the institution's prior express consent.

The Standard Protocol Items: Recommendations for Interventional Trials reporting guidelines and checklist have been observed in the publication of this study protocol.[18] Data from the statistical analysis and from the protocol will be made publicly available on its most possible extension, enhancing transparency, reproducibility and interpretation of trial results. The project coordinator will participate in the authorship of the paper as well as others selected on the basis of their contribution. Investigators will not be precluded or limited from publishing the results of the study.[19] The acknowledgement section will be as explicit as possible with any other contribution(s).

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Contributors FC and MF conceived the study and its implementation. AA, AM, CC and GQ designed the protocol. FC, WR, GQ and AG wrote the manuscript with the inputs from all the team. All authors contributed to the refinement of the study protocol and approved the final manuscript.

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Competing interests None declared.

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 applicable.

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