B.L.U.R. STUDY

(BLUE LIGHT FOR ULCERS REDUCTION)

Multi-centreStudy on the Effectiveness of Treatment With a Blue Light Medical Device(EmoLED) in the Reduction of Ulcer Surface in 10 Weeks

PROTOCOL VERSION 3.0 OF 21/11/2017

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1. INTRODUCTION

Wound healing is a complex and dynamic biological process consisting of a series of organised stages, including coagulation, inflammation, matrix deposition, angiogenesis, proliferation, cell remodelling and wound contraction (1, 2). To complete each stage of the process, complex interactions must take place between several biological factors, such as growth factors and proteases, matrix components and various types of cell, such as platelets, macrophages, fibroblasts and endothelial cells (3). The interaction between these processes as a whole results in restored tissue integrity and functional healing (4). Although it is generally acknowledged that most acute wounds can heal following the ordered sequence of events described above within 2-4 weeks, sometimes, due to changes occurring during the natural healing process, a pathological scar (either a hypertrophic scar or a keloid scar) can form. In addition, the healing process may be blocked at various stages of the regenerative process, which prevents the affected area from healing, giving rise to the development of chronic skin wounds (ulcers[1]). Many factors can contribute to the occurrence of these events, some of which are already known, whereas others such as diabetes, venous or arterial disease, infections, metabolic disorders and advanced age are still being investigated.

Therefore, the proper management and treatment of wounds after surgery, trauma or a pathological process plays an important role in the healing process, not only to prevent the onset of infections or other complications but also to accelerate the wound healing process and limit scarring as much as possible.

Although much work has already been done to fully understand the processes governing this extremely important biological function, many aspects remain unclear. Although it is true that technological progress has led to the creation of increasingly sophisticated treatments, there is also a need to find new methods that can further improve the healing process, irrespective of the cause of the skin lesion, reducing the pain and discomfort associated with the medication itself and potentially shortening the healing time, thereby reducing the associated costs.

The purpose of this study is to evaluate the efficacy of the EmoLED device in promoting the healing of skin ulcers. As it is an innovative device, data relating to its use in humans has not yet been reported in the literature. A first pilot study investigated the use of the above-mentioned device on skin sampling areas in patients treated by the Centro Grandi Ustioni [Severe Burn Centre] of the AOUP (Azienda Ospedaliero-Universitaria Pisana [Pisana University Hospital]). This study demonstrated the clinical safety of the device as well as its non-inferiority compared to other types of advanced treatment.

This study will consider skin ulcers of various aetiologies. Depending on the size of the wound or the presence of multiple wounds, the EmoLED device will be used to treat either the whole wound or half of the wound.

How the treatment will be applied will be explained in detail below. However, in short, treatment with EmoLED consists of irradiating the wound (or part of the wound) for 60 seconds with the light emitted by the device. This treatment is used as an adjuvant to standard therapy.

This treatment does not interfere with other systemic therapies that may be being administered, nor does it involve any additional risk compared to SOC. The potential benefit is reduced healing time, with the consequent indirect benefits that this entails.

1.1 Operating principle of the EmoLED medical device in supporting wound healing

The EmoLED device is a medical device designed to support wound healing, whose operation is based on LEDs that emit blue light. It has not yet attained CE marking as its certification process is pending.

Blue light was chosen based on Anderson and Parrish's theory of selective photothermolysis, according to which selective heating of a target chromophore is achieved when the source wavelength – our LEDs – is preferentially absorbed by the chromophore – Protoporphyrin IX of the haem group and Cytochrome c group.

[1] According to Welfare reports, 1% of adults in Italy suffer from leg ulcers. This percentage increases to 3.6% in adults over the age of 65 years.

Regarding the use of light in medicine, the scientific literature is abound with data and evidence on its use, both for the treatment of acne (5) and psoriasis (6) and on its efficacy in accelerating the healing process in animal models of induced wounds (7), (12).

When considering the skin chromophore absorption coefficients within the range of the visible spectrum, it is observed that haemoglobin reaches its maximum absorption peak within the blue range: at around 410 nm for oxyhaemoglobin (HbO_2) and around 430 nm for deoxyhaemoglobin (deoxy-Hb). This particular property is exploited to induce a localised temperature increase in order to stimulate a rapid coagulation effect. The use of the selected wavelength range also ensures that the thermal effect is focussed entirely on the areas where haemoglobin is present, thereby limiting the effect on the healthy tissues surrounding the wound through reduced absorption by the other skin chromophores (11).
A review of the relevant literature shows that vanilloid receptors, which are sensitive to different temperature ranges and are expressed by various cell types present in the treated tissues (8), (9), (10), may be activated by the thermal action of the EmoLED device on haemoglobin. Of the various receptors that may be involved, it is worth mentioning theTRPV1, TRPV3 and TRPM7 channel receptors, which are co-expressed and mutually regulated. The activation of these receptors by thermal stimulation triggers a signal transduction pathway in the cells. This pathway is involved in phenomena such as mast cell degranulation as well as the release of pro-inflammatory mediators and the production of collagen, ultimately accelerating the overall healing mechanism. Another pathway through which the EmoLED device can act is the mitochondrial electron transport chain. More precisely, EmoLED can act on the last two complexes containing Cytochrome c, which is also sensitive to the visible light emitted by the device. The resulting effect is an enhancement of this process as well as an increase in ATP production related to the development of an electron transport chain-dependent proton gradient. Increased ATP production results in an increase in the amount of energy available to cells, which can enhance their metabolic activity. This process is necessary for wound repair because healing involves the simultaneous activation of different cell types and additional energy consumption by the body.

The device has been manufactured and programmed to provide a power density of approximately 5 J/cm^2 within an acceptable treatment time both for doctors and patients, corresponding to 60 seconds for every 5 cm x 5 cm area to be treated.
Treatment duration was also chosen based on the data reported in the literature on thermal injury. The temperature generated by the treatment on the treated area ranges from 45°C to 50°C, which is optimal for stimulating the induction of the desired reversible and physiological phenomena.
A table detailing the temperature-induced physiological phenomena is provided on page 78 of (13) and attached hereto:

Table 3.6. Thermal effects of laser radiation

Temperature	Biological effect
37°C	Normal
45°C	Hyperthermia
50°C	Reduction in enzyme activity, Cell immobility
60°C	Denaturation of proteins and collagen, Coagulation
80°C	Permeabilization of membranes
100°C	Vaporization, Thermal decomposition (ablation)
> 150°C	Carbonization
> 300°C	Melting

The same source shows a curve demonstrating a link between the duration of thermal stimulation and the degree of reversibility of the process induced by thermal stimulation.

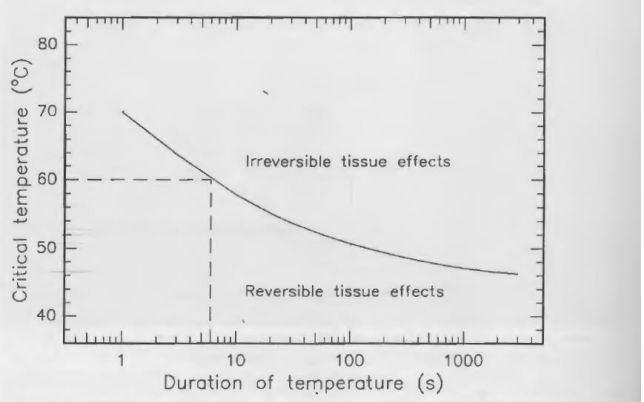

Fig. 3.21. Critical temperatures for the occurrence of cell necrosis. Data according to Henriques (1947) and Eichler and Seiler (1991)

This finding concerning the temperature-induced physiological effects constituted the foundation for the definition of the treatment parameters.
In addition to the studies on thermal influence, the choice of treatment duration is based on a finite element model (14) which correlates optical power to treatment duration and the final temperature induced by the treatment with the device. The initial model led to the identification of the optimal working conditions. These were subsequently tested in an animal model study.
As reported in the article, the animal study evaluated treatment efficacy adjusted to the standard represented by an abrasion produced on the back of the animal using a mechanical abrasion instrument. No thermal injury was observed in the tissue surrounding the wound and it was confirmed that a single treatment was sufficient to trigger and accelerate the wound healing process.

The above-mentioned mechanisms are in line with the findings directly observed during a preclinical study of an animal model as part of the LighTPatcH project within the 2012 BiophotonicsPlus call, which used a fibre optic version of the EmoLED device to treat abrasions produced on the back of male albino CD-1 mice.

PRE-CLINICAL ANIMAL MODEL STUDY PROTOCOL
Mice were anaesthetised with ketamine (80-100 mg/kg, IP) and xylazine (10 mg/kg) intraperitoneally and placed on a warming pad heated to 37.5°C to prevent further anaesthesia-induced stress. Their backs were shaved using an electric razor. Each animal received two wounds, one rostral and one caudal, inflicted by an abrasor. The two wounds, measuring 1 cm in diameter, were marked out and delimited. One of the wounds was treated with the EmoLED device. Subsequently, both of them were treated with Streptosil (neomycin) (topical preparation) to prevent the onset of infections.
The treatment with the EmoLED device lasted 30 seconds in all cases (the EmoLED device we intend to test in humans can treat round areas up to 2 cm in diameter; for larger wounds, a 30-second treatment will have to be repeated for each sub-area until the whole area is treated).
After the treatment, the animals were housed in individual thermostatic cages until they awoke from anaesthesia. During the healing process, prior to sacrificing them, the animals were housed in individual cages under controlled temperature and light conditions (21-24°C and 12/12 hours of dark and light cycles) and supplied with standard water and rodent pellets ad libitum.

PRECLINICAL ANIMAL MODEL STUDY OBSERVATIONS
The observations reported below were extrapolated from tests performed on biopsies taken at specific time points. After the treatment, each animal was observed over a given follow-up period before being sacrificed. The areas to be studied were collected and embedded in a compound suitable for cryosectioning (frozen section). They were then used both for histological and immunohistochemical analyses.
A large number of observations were collected. They are reported below by making reference to the observed phenomenon.

Inflammatory infiltrate: based on the data obtained 0, 1, 3, 6, 9, 12, 18 and 24 hours after inflicting the wound, it is noted that within the first few hours the amount of inflammatory infiltrate in the areas treated with the EmoLED device is higher compared to the untreated wound. The difference becomes smaller between 9 and 18 hours until, after 24 hours, the situation is reversed and the amount of inflammatory infiltrate in the untreated wound is higher than that in the treated wound.
Mast cells and Mast Cell Degranulation Index: although no particular differences in the number of mast cells were found between the treated and untreated samples, this was not the case when mast cell activation was studied: in fact, potent degranulation was observed in the treated sample at 3 hours, which was not seen in the wound not treated with the EmoLED device.
Macrophage populations: observations made 0, 3, 6, 9, 18 and 24 hours after inflicting the wound on the populations of M1 macrophages (proinflammatory) and M2 macrophages (pro-healing) show that the anti-inflammatory phase begins 6 hours after inflicting the wound in animals treated with the EmoLED device, whereas it is necessary to wait 18 hours for the inflammatory phase to end with the standard treatment. Moreover, after 18 and 24 hours, both the M1 and M2 populations are comparable in the treated and untreated samples, demonstrating that treatment with the EmoLED device does not induce responses other than normal physiological reactions.

Based on these studies, it can be concluded that the EmoLED device exerts its effect within the first few hours after wound infliction (and therefore within the first few hours after treatment). Our hypothesis is that treatment with the device exerts its effect early in the inflammatory phase of healing and promotes healing both in terms of time (observable based on the data obtained from the inflammatory infiltrate study) and quality, evident from the significant mast cell activation, resulting in greater histamine release, causing increased activation of various mediators and cell types that may be involved in the healing process.

IN-HUMAN ACUTE WOUND STUDY OBSERVATIONS AND ULCER CASE STUDY
The safety and efficacy clinical validation to obtain the CE marking for the EmoLED device for the treatment of acute surgical wounds is ongoing. **In particular, the acute observations, whose detailed results are provided in the attached final Report on the clinical trial, have once again demonstrated the device's safety and efficacy in shortening the healing time of spontaneously healing wounds.**
Isolated observations in patients with chronic lesions, of various aetiologies, have shown that the device is capable of unblocking the healing process, thus allowing the wound to progress beyond the inflammatory phase by inducing re-epithelialisation and wound closure. Observations made using a thermal camera have also shown that, due to the compromised condition of the tissues surrounding the ulcers, the baseline temperature of the limb is lower than normal and that both during and after treatment with the EmoLED device, the temperature never exceeds 40°C.

2. STUDY OBJECTIVES

The aim of this clinical study is to determine whether the proposed treatment represents a valid and significant treatment for ulcers by evaluating the percentage reduction of the area of the wound over a 10-week observation period and comparing the reduction of the area of the wound (or part of the wound), both on the same patient and within the entire group, treated with the EmoLED device, versus the control wound (or part of the wound) treated with a standard method. Over the 10-week period following their recruitment, the patients will continue to take their usual topical therapy and attend the weekly visits. Patients will be monitored:

- until healing, if this occurs before week 10,
- until week 10 in the event of incomplete healing.

Additionally, given that the device does not yet have CE marking, reports and assessments on the safety and usability of the device will be complied by the medical staff during the study.

3. ENROLMENT

A total of 90 subjects will be recruited by the facilities involved in this Clinical Study. Both hospitalised patients and outpatients receiving treatment will be considered for inclusion in this Study.
Patients will be assessed using a standard procedure that includes taking a medical history and a physical examination.

3.1 Inclusion criteria

- Subjects with venous, arterial or mixed skin ulcers and surgical wound dehiscence (18), (19);
- Presence of multiple similar wounds or one wound measuring[2] at least 5 cm;
- Men and women ≥ 18 years of age;
- Patients must be able to understand the aims of the Clinical Study and provide their informed consent in writing;
- Chronicity of the wound: at least 8 weeks.

3.2 Exclusion Criteria

The following will not be included in this study:

- Patients who participated in a clinical study on skin ulcer healing during the previous month;
- Patients who are unable to understand the aims and the objectives of the study*;*
- Patients with neoplastic ulcers;
- Patients with decubitus ulcers;
- Patients with diabetic foot ulcers;
- Patients with clearly infected ulcers;
- Patients with ulcers caused by critical ischaemia;
- Patients with a history of self-harm that can purposely alter the healing process;
- Patients with psychiatric disorders;
- Pregnant or breastfeeding women[3];
- Patients with neoplasms or other diseases involving the use of cytostatic or immunosuppressive drugs;
- Patients with a short life expectancy.

In order for subjects to be eligible for inclusion in the treatment phase, all inclusion and exclusion criteria must be met before enrolment. Any concomitant drug therapies must be maintained.

3.3 Subject recruitment and screening

Enrollable patients will be selected by the Principal Investigator or his/her delegate based on the study inclusion and exclusion criteria. Once it has been verified that patients meet the study inclusion and exclusion criteria, the Principal Investigator or his/her delegate will ask the patients to consent to the use of the EmoLED device for treatment, after briefly explaining to them the key features of the device and the expected effect. If patients agree, they will enter the treatment phase. At the same time, patients will agree to return to the facility every week over the required observation period.

Patient registration in the identification register

Each trial site must keep a register of all subjects enrolled in the clinical trial and assign an identification code, an alternative subject identification or contact information. (UNI EN ISO 14155:2012 para. 6.5.2)

[2] In the event of multiple wounds, one wound will be treated with the EmoLED device and one will be chosen as the control wound; large wounds will be divided into two halves, one of which will be the control wound. This will reduce the variables associated with the inter-individual variability of skin lesions, thereby increasing the power of the test.

[3] Pregnancy and breastfeeding will be verified based on the patients' declaration.

To comply with this specific requirement, the Principal Investigator will create, update and keep the Patient Identification Register.
The Patient Identification Register will contain the personal details of the patients who have given their consent to participate in the study, as well as the enrolment date and the patient code, comprising the acronym of the province of the facility followed by a three-digit sequential numerical code.
The patient code will be indicated in the Patient Case Report Form as Name (site ID code) and Surname (number) in order to create a database of photographic records.
Only the Principal Investigator and, at the discretion of the Principal Investigator, the members of his/her team will be aware of the identity of the patients enrolled in the study.
If a patient withdraws from the study, the Principal Investigator or one of his/her delegates will record the event in the Identification Register and on the Case Report Form and will archive everything.

Patient Enrolment Form

For each new patient, the Principal Investigator or his/her delegate will complete the Patient Enrolment Form with information such as patient identification (Name and Surname will be the same as in the Case Report Form, i.e., site ID code and number), age, gender, root cause of the ulcer, any concomitant conditions, any pharmacological therapies, date of onset of ulcers and recruitment date.
The second part of the form contains a table for recording the visit date, the patients' general health and the data collected at each visit.

Recording of clinical data

The data pertaining to the evaluation of the study endpoints will be acquired at specific time points as indicated in the Protocol and in the Study Case Report Form.
The parameters recorded on the Case Report Form will be acquired and maintained in paper format for the entire duration of the trial and stored both in paper and digital format, after scanning the Case Report Form, once a patient has completed the study; photographic images of wounds will be acquired, maintained and stored in digital format for the entire duration of the trial and afterwards.
The archiving code follows the patient recruitment code and the time of acquisition.

Case Report Form

The same Case Report Form will be used for all patients. It consists of many columns to be completed, one for each observation time point. At each observation time point, the doctor will mark the value chosen for each parameter with an “X” and the most appropriate description.
The Case Report Form will include a “Notes” section for recording additional information not initially envisaged that may be useful during the data analysis phase (e.g., patient's ethnic origin, special environmental conditions, patient's specific health conditions such as influenza or others, etc.).
Following the patient's completion of or withdrawal from the study, the Case Report Form will be scanned and archived.

Centralised database

The Principal Investigator of each site will send the Enrolment Form of any new recruited patients as well as the photographic records of the follow-up patients to the Project Manager on a weekly basis. The Case Report Forms of all patients who leave the study, whether healed or identified as drop-outs, will also be submitted.
Based on this data, the Project Manager will create a single database containing the data of all patients recruited, updated every week with the latest available data, accessible to the Principal Investigators. This database will allow the Investigators to monitor both the remaining number of patients yet to be recruited and the progress of the study.

4. STUDY DESIGN

4.1 General design

This clinical study will be a multicentre, prospective, controlled study, whose aim is to evaluate the clinical efficacy of a battery-powered portable device that uses blue LEDs.
As described in greater detail in the statistical analysis section, at least a 20% difference between treated and untreated wounds is expected to be observed in the same patient within the specified observation period.

For safety, the operator will be equipped with UV protection glasses. The housing of the device is made of biocompatible material that can be disinfected with detergents commonly used in hospitals in order to prevent contamination.
The device passed all safety tests (electrical, electromagnetic and photobiological) required by current regulations on medical devices, as well as a precise risk and usability analysis, and was found to be suited to the intended use.

The treatment, which is provided in addition to the patient's standard therapy, will be performed at each visit for 60 seconds on each 5-cm diameter sub-area of the selected wound or part of it.
Therefore, for multiple wounds, one will be treated with the EmoLED device and one will be selected as a control wound; very large wounds will be divided into two halves and one of the two will be the control wound. This will reduce any variability associated with randomised clinical trials (RCTs) on skin ulcer conditions.

4.1.1 Selection of the treatment area.
All wounds will be cleaned with saline solution and, if there is slough/black eschar, surgical debridement will be performed using a scalpel. Only at this point will treatment with the EmoLED device take place.
If a patient has more than one wound and all wounds at enrolment are found to have a **diameter of less than 5 cm**, the worst of the wounds will be treated entirely with the EmoLED device, whereas the others will serve as control wounds. Progression of all wounds will be evaluated over the ten-week study duration.
By contrast, if a wound at recruitment has a **diameter greater than 5 cm,** the wound will be divided into two halves along the longest side, and only one of the two halves will be treated. The other half of the wound will be dressed with a multi-layered sterile dressing. The division line between the two halves of the wound will be marked with a permanent marker, which will be reapplied at each visit, or whenever it begins to fade, to prevent loss of the reference marks.
If, at recruitment, a patient has more than one wound with a **diameter greater than 5 cm**, all wounds will be divided into two halves along the longest side and treated as described above.
After treatment with the EmoLED device, the wound will be dressed with a hydrofibre dressing. If clinical signs of infection occur, the wound will be dressed with a hydrofibre dressing with silver. The limb will then be bandaged with a compression bandage.

4.1.2 Standard treatment and data recording.
Any wounds or parts of wounds that are not treated with the EmoLED device will be medicated using the standard treatment consisting of a hydrofibre dressing or hydrofibre dressing with silver (as described above) and compression therapy. Ultimately, the treatment differs only for the fact that the EmoLED device is used in accordance with the procedure described above, while the rest of the treatment remains unchanged.
Following enrolment and the signing of the Informed Consent Form, the pertinent data from each patient will be recorded in the respective Case Report Form according to the following schedule: W0 (Week 0: enrolment and first treatment), W1, W2, W3 ... to W10, as per the routine visits over the 10-week period from enrolment.
Patients may miss a maximum of two non-consecutive follow-up visits over the 10-week period, starting from visit 5. Patients will be excluded from the study if consecutive visits are missed.

The parameters used to define the study outcomes will be based on:

- photographic images of the wounds treated with the EmoLED device as well as of control wounds
- Case Report Form completed by the doctor during the visits.

4.2 Primary endpoint of the study

The primary endpoint of the study will be a comparison of the outcomes in terms of percentage reduction of the area of the wound treated with the EmoLED device versus the wound treated with SOC at W10, both the difference in the individual patient and within the entire group of 90 recruited patients.

4.3 Secondary endpoints

The following secondary endpoints were identified for this study:

1. Treatment safety.
2. Outcomes comparison in terms of the percentage reduction of the area of the wound treated with the EmoLED device versus the wound treated with SOC only within the ten-week observational period.
3. Evaluation of difference in healing time between the two areas.
4. Pain reduction (with pre-treatment assessment on a 0-10 VAS)
5. Depending on the patient's willingness to continue to go to the study site, the observation of any unhealed area(s) over the 10-week period can be extended until complete healing or up to 20 weeks.

5. ADVERSE EVENTS

Although the available information and the physical and performance characteristics of the device do not suggest the possibility of adverse events, this section of the protocol considers the possibility that such events might occur.

An Adverse Event (AE) is any unfavourable change in health, or side effect, or unexpected benefit that occurs in a subject involved in a clinical study while the patient receives the treatment or within a specified period of time following completion of the therapy, which may not necessarily have a causal relationship with such treatment or clinical trial. An adverse event may therefore be any unfavourable and/or unexpected sign or symptom (including abnormal findings in laboratory tests), or a disease or condition temporally associated with the use of a medicinal product (being investigated during the trial) or an investigational device, irrespective of whether or not it is related to such medicinal product or device (being investigated during the trial).

The morbidity of each subject will be monitored throughout the study period. Any signs exhibited by patients will be observed during each study visit; patients will be prompted to report possible symptoms through direct questioning; for example: "How have you been feeling since your last visit?". Moreover, subjects will also be encouraged to spontaneously report any adverse events that may occur during the Study.

Any clinically significant anomaly found during the Study will be monitored and observed until normalisation, or until it can be clinically explained.

5.1 Serious Adverse Events

A Serious Adverse Event (SAE) is defined as any unfavourable medical event which, at any dose, results in one of the following:

- Leads to patient's death;
- May lead to a fatal outcome in the short term;
- Leads to subject's hospitalisation or prolonged hospitalisation;
- Causes significant or persistent disability/incapacity;
- Another significant medical event.

Significant medical events that do not lead to the patient's death, or are not life-threatening, or do not require hospitalisation, may nevertheless be considered serious adverse events when, based on appropriate medical judgement, they may pose a risk to the subject's health or may require medical or surgical intervention to prevent one of the *outcomes* described above.
Hospitalisation is defined as the subject's stay (usually for at least one night) in a hospital or Accident and Emergency Room for observation and/or treatment. When it is uncertain whether hospitalisation occurred or was necessary, the adverse event must be considered serious.
Hospitalisation for elective surgery or for *routine* clinical procedures that are not the consequence of an adverse event should not be considered an adverse event. If an unfavourable event is reported during the procedure, it must be reported as an adverse event; whether this is "serious" or "non-serious" will be decided based on the usual criteria.

5.2 Reporting of Adverse Events and Serious Adverse Events
All adverse events reported by the subject or observed by the investigator or by a member of his/her *team* will be reported in the Serious Adverse Event Form. The following information must be collected for each adverse event:

- onset and resolution date and time (duration);
- seriousness (defined subsequently in the text);
- any required treatments or interventions adopted*;*
- *outcomes;*
- relationship to the product used in the trial (defined subsequently in the text);
- if the adverse event has caused the subject to withdraw from the Study.

All adverse events must be recorded using the accepted diagnoses, where possible. Any abnormal laboratory results, vital signs or abnormalities detected at the physical examination must not be recorded as adverse events if it is known that they are a symptom or sign of a syndrome or a diagnosis already reported as such. For example, ST-segment elevation detected by ECG and increased serum creatine phosphokinase are both known signs of myocardial infarction; therefore, they need not be reported if myocardial infarction was already reported as an adverse event. However, if both occur separately and no myocardial infarction was diagnosed, then this event must be reported as an adverse event.
Seriousness categories and the causal relationship of adverse events must be defined using the parameters listed below:

<u>Seriousness:</u>

mild	easily tolerated, does not hinder normal activities of daily living
moderate	partially hinders activities of daily living
serious	fully hinders all activities of daily living

<u>Relationship with the Study procedures:</u>

Not related: the temporal relationship between the adverse event and the use of the Study product is such that a reasonable relationship between the two is very unlikely.

Possibly related: the adverse event occurs in a time sequence consistent with the start of use of the Study product, but it may also be attributed to the subject's clinical status or to the Study procedures/requirements.

Definitely related: the adverse event occurs in a time sequence consistent with the start of use of the Study product, disappears after its discontinuation and appears again when it is resumed.

In addition to being recorded in the Case Report Form (*CRF*), serious adverse events (SAEs), which include suspected unexpected serious adverse reactions (SUSARs), must be reported by the investigator on the "Serious Adverse Event Report Form". This form must be completed and sent to the Study Coordinator immediately, i.e. within 24 hours from the detection of the SAE by the research staff. The original must be sent and a copy stored at the trial site.

5.3 Safety follow-up of subjects who have withdrawn from the Study

Subjects who have withdrawn from the trial after treatment with the Study device for any reason will undergo ongoing monitoring of any existing adverse events until the findings of the tests related to the adverse event and required by SOCs have returned to baseline values, or until the investigator has verified that these events are no longer clinically significant.
Subjects who exhibit adverse events up to thirty days after the last visit or the last *follow-up* (whichever occurs last) will be monitored until the findings of their tests have returned to baseline values, or until the investigator has verified that the results are no longer clinically significant.

5.4 Safety follow-up of subjects who have completed the Study

All subjects with adverse events when they complete the Study will be monitored until the findings of their tests have returned to baseline values, or when no further changes are expected, or until the investigator has verified that the results are no longer clinically significant.

6. STATISTICAL PLAN

Ninety subjects will be enrolled in this study. Each subject is part of both the control group and the treatment group.
Given that each patient will both receive the treatment and will also be a control subject, both groups will contain the same number of subjects, even if some of the subjects withdraw from the study. A comparative study of the subjects' demographic data and other baseline characteristics will also not be necessary as the two groups will be perfectly superimposable.

Treatment will be administered immediately after the cleaning and any necessary debridement of the complete wound. The area of the wound that should not be treated with the EmoLED device will be duly marked and covered with different layers of sterile dressing; this facilitates treatment standardisation and uniformity, thereby limiting any variability; it will also enable the treated, untreated and adjacent areas of each subject to be coherently defined.

The aim of the statistical analysis will be to establish the efficacy of the treatment with the EmoLED device; the significance ($p<0.05$) of any difference observed in the healing process between treated and untreated areas will be evaluated. Specifically, the primary endpoint of the study is the percentage reduction of the area of the wound treated with the EmoLED device versus the wound not treated with the EmoLED device at W10.
The secondary endpoints are:

1. Treatment safety.
2. Outcomes comparison in terms of the percentage reduction of the area of the wound treated with the EmoLED device versus the wound treated with SOC only within the ten-week observational period.
3. Evaluation of difference in healing time between the two areas.
4. Pain reduction (with pre-treatment assessment on a 0-10 VAS)
5. (OPTIONAL) Depending on the patient's willingness to continue to go to the study site, the observation of any unhealed area(s) over the 10-week period can be extended until complete healing or up to 20 weeks.

Estimation of sample size

The considerations underlying the choice of the model described below were drawn from a review of the literature on leg ulcer therapies and healing dynamics as well as on past clinical experience data obtained by the sites involved in the study with the treatment in question.

The most significant finding yielded by the analysis of the clinical data in our possession is an expected 30% variability in the percentage reduction of the area of the wound at week 10. This value was confirmed by [15], where it was used to estimate the sample size. On the other hand, [16] found the standard deviation of the percentage reduction of the area of the wound to be 53% in the worst case and limited to venous ulcers only.

The analysis for the calculation of the sample size is based on:

- the clinical objective, which is represented by the percentage reduction of the area of the wound at W10
- a non-parametric statistical instrument for paired data, namely the Wilcoxon signed-rank test
- an a priori hypothesis of expected variability of 50% (conservative value)
- an expected 20% mean difference in the percentage reduction between the treated and the non-treated area
- a statistical power of 80% (β=0.2) and α=0.05) will be considered
- a unilateral alternative hypothesis: the EmoLED treatment does not result in worsened outcome
- A correlation coefficient between percentage reductions of the treated and untreated area of 0.1, 0.3, 0.5 and 0.7
- A method for estimating the sample size based on asymptotic relative efficiency, compared to the t-test (minARE)

	Estimate 1	Estimate 2	Estimate 3	Estimate 4
β	0.2	0.2	0.2	0.2
α	0.05	0.05	0.05	0.05
Diff. in % wound area reduction after 70 days	20%	20%	20%	20%
ρ	0.1	0.3	0.5	0.7
n	83	65	47	29

The higher estimate is that relating to case 1, whose sample size is 83 subjects, which served as the basis for the choice of the number of subjects to be recruited, taking into account possible loss of subjects during treatment.

Statistical analysis of the primary endpoint.

A Wilcoxon signed-rank test with a significance level of 0.05 will be performed to evaluate the efficacy of use of the device. This test allows paired data to be handled and is robust enough to evaluate variability in observation distribution. A population analysis, as well as a per-protocol analysis (PP), will be performed for the subjects who complete the study.

Statistical analysis of secondary endpoints.

1. Treatment safety.

Adverse event reports will be collected, analysed and managed according to the criteria indicated in 5.2. The most appropriate statistical instrument will be chosen for analysis based on the number and type of adverse events reported.

2. Outcomes comparison as percentage reduction of the area of the wound treated with the EmoLED device versus the wound treated with SOC alone during the 10-week observation period.

Both the areas of the wound treated with the EmoLED device and those not treated with the EmoLED device, as measured on the required observation days, will be considered. A statistical analysis of paired data will be

performed using repeated measurements. In particular, both a comparison of the measurements obtained on different days, separately, and an analysis that will take into account the global trend over the observation period, will be performed.
Tests for data paired to pairs of measurements obtained on different observation days will be performed in the first case. These tests will be applied separately to the various days, evaluating any significant differences in both treated and untreated areas. The adopted statistical test will be either a parametric (t-test) or a non-parametric test (Wilcoxon signed-rank test) depending on the observed data distribution. The statistical significance will be corrected for multiple comparisons (e.g., given the possibility of a positive correlation between the identified measurements, Benjamini-Hochberg correction will be considered).
In the second case, the area under the curve (AUC) will be evaluated for each subject and in each area in order to obtain information on possible differences between changes in the treated and untreated areas of the wound. Either parametric or non-parametric paired data tests (depending on parameter distribution) will be performed in order to evaluate any differences in the trends of this parameter summarising the healing dynamics both in the treated and untreated areas.
The mean value observed in the remaining subjects will be used for both approaches if there is any missing data. A sensitivity analysis will be performed using both the best value and the worst value observed in the subjects.

3. Evaluation of the difference in healing time between the two areas.
A survival analysis will be performed based on the Akritas test applied to the Kaplan-Meier curve. This type of analysis is used to evaluate the time from observation to the event of interest (time-to-event). In particular, in this case, the event will be a percentage reduction of the area of the wound of ≥70% at W10. This approach will allow both paired data and the possibility that some of the subjects may be non-adherent to the protocol and withdraw from the study to be managed. A curve will be constructed for each group, each corresponding to the analysis of the two areas, both that treated with the EmoLED device and that not treated with the EmoLED device. The Akritas test is based on a transformation into ranked "survival" times, understood as time to event of interest (time-to-event). In particular, in this case, the event will be a percentage reduction of the area of the wound of at least 70% at W10. Ranked observations will be transformed according to a model that takes into account the possibility that they may be truncated or censored.

4. Pain reduction (pre-treatment assessment on a 0-10 VAS).
A non-parametric analysis will be performed using the Friedman test, which can be considered the non-parametric equivalent of the ANOVA test for repeated measurements.

5. (OPTIONAL) Depending on the patient's willingness to continue to go to the study site, the observation of any unhealed area(s) over the 10-week period can be extended until complete healing or up to 20 weeks.
The analysis will be performed as for endpoint 2.

7. CONFIDENTIALITY OF INFORMATION

All information collected during the Study will be considered strictly confidential.
Patients' consent to the recording of their personal data will be requested at enrolment. However, the patients' Case Report Forms will contain only two patient identification codes, i.e. their initials and date of birth. In order to ensure the proper traceability of all participating subjects, the study number and the name of the site will also be used.

7.1 Archiving

At the end of the study, the data will be archived at the Coordinating Site of the study for a minimum of ten years, adopting all appropriate security measures. Should a patient withdraw their consent to the processing of their personal data, this data will be immediately destroyed, guaranteeing total confidentiality.

Additionally, each investigator will keep a copy of the Study documentation for a minimum of two years following its completion.

8. REQUIRED MONITORING

To ensure compliance with the ICH-GCP guidelines, the Coordinator will be responsible for ensuring that the Study is conducted in full compliance with the Standard Operating Procedures, the Protocol and any other written instructions.

The Coordinator's main responsibilities include: ensuring that the Protocol is complied with, ensuring accuracy and completeness in data recording and reporting, and verifying that the informed consent has been obtained from and recorded for all subjects before their participation in the Study.

The Coordinator will contact and meet the Investigators on a regular basis throughout the duration of the Study. The Coordinator will be entitled to monitor and verify all Study-related documents (Case Report Forms or CRFs and other relevant source data documents) to ensure both compliance with the Protocol and that the recorded data is complete, consistent and accurate.

9. ETHICAL CONSIDERATIONS

This Study will be conducted in accordance with the principles set out in the Declaration of Helsinki and all applicable legislation and local regulations on clinical trials conducted on research subjects.

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