"MOJITO"

Clinical Study to Identify Biological Markers to Predict Wound Healing in Patients with Chronic Venous Ulceration of the Lower Limb

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Clinical Queries

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Sponsor

Imperial College London is the main research Sponsor for this study. For further information regarding the sponsorship conditions, please contact the Head of Regulatory Compliance at:

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Funder

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This Protocol will describe the MOJITO study and provides information about procedures for entering participants Every care was taken in its drafting, but corrections or amendments may be necessary/ These will be circulated to investigators in the study. Problems relating to this study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlines in the NHS Research Governance Framwork for Health and Social Care (2nd edition). It will be conducted in compliance with the protocl, the Data Protection Act and other regulatory requirements as appropriate.

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1. Background and Objectives

Chronic venous ulceration of the lower limb represents a significant problem to patients and providers of healthcare worldwide. It is estimated that 0.3% of the population suffer from an open venous ulcer and that 1% of the population have either an open ulcer or chronic venous ulcer (1). In the UK, the cost of treating an unhealed ulcer is approximately £1300 a year (2).

The established treatment of chronic venous ulceration in the United Kingdom is to provide a course of compression bandaging <reference>. However, despite the use of this treatment recurrence at one year has been shown by one study to be 17% (3). In addition, some ulcers will not heal with compression (3).

When dealing with chronic venous ulceration it may be useful to visualise the process of a failure of normal healing to progress through its well defined pathway. Multiple groups have attempted to unpick the mechanism of failed wound healing. However, our knowledge of this area is incomplete and requires further clarification.

1.1 Objectives

We seek to further examine the biology of chronic venous ulceration. Our rationale for this study is:

1. Can we effectively predict from biological markers in whom standard therapy will not culminate in wound healing?

2. Can potential therapeutic targets be identified?

3. What changes occur in the biological profile of a chronic venous ulcer over various points in time?

Our belief is that the potential benefits of this study to wider healthcare community may be in effectively stratifying patients with chronic venous ulceration by risk of treatment failure. This may allow resources to be concentrated on high risk patients.

In addition, identifying therapeutic targets may in the future reduce the proportion of patients in whom standard treatment fails or ulcers recur.

Study of the same patients over time will help us understand the process of healing in chronic venous ulceration and determine the differences in patients who ultimately heal versus those who do not.

2. Eligibility and Inclusion

- Forty patients to be recruited from Vascular Surgery Clinics at Imperial College NHS Trust, and Cambridge University Hospitals NHS Foundation Trust.
- Male or female over the age of 18 years
- Chronic venous ulceration – Defined as wound of greater than four weeks in duration between the foot and the ankle with an Ankle Brachial Pressure Index greater than 0.85.
- Ulceration present for at least four weeks.
- Colour venous duplex evidence of chronic venous insufficiency showing either reflux or obstruction.

3. Exclusion Criteria

- Acute infection in the studied lower limb within the last four weeks
- History of malignancy in the lower limb to be studied
- History of connective tissue disease

- Patients on medications that can cause immunosuppression – Corticosteroids, chemotherapy or radiotherapy for cancer and recombinant immunological medications.

4. Study Design and Methodology

Forty patients who fulfil the criteria for the study will be recruited from Vascular Surgery clinics in hospitals under the auspices of Imperial NHS Trust in London and Cambridge University Hospitals NHS Foundation Trust in Cambridge.

In summary, this study is a prospective cohort study of patients with venous leg ulceration with samples of blood, urine and venous leg ulcer fluid collected over multiple time points over an initial twenty-week period with standard treatment for leg ulceration. In patients not healed fully at twenty weeks, they will be offered an Urgostart® dressing and further assessments carried out to the end of follow up at twenty-eight weeks.

The study will take place between August 2013 and February 2015.

4.1 Procedures and Assessments

4.1.1 Initial Assessment

- A full history and examination will be completed.
- Venous severity scoring (4).
- CEAP (Clinical-etiologic-anatomy-pathophysiology) scoring (5).
- Colour venous duplex to establish integrity of the venous system.
- Ankle brachial pressure index.
- Routine blood tests: Full blood count, urea, electrolytes, glucose, glycosylated haemoglobin (if patient suffers from diabetes).
- Assessment of wound healing: This will be performed using digital planimetry by which electronic representations of the ulcer are formed and the area calculated.
- Wound ulcer fluid – Two techniques performed simultaneously.
    1. “Occlusive dressing technique” (6). This technique involves the placement of a clear adherent dressing over the ulcer. After ninety minutes in a seated position. The dressing is pierced with a 21 gauge needle and any accumulated fluid is sampled.
    2. “Filter disc technique” (7). This involves placement of filter discs on top of the ulcer bed separated by a permeable transparent dressing to filter out solid material.
- Urine sampling: A mid-stream urinary sample shall be requested from the patient in a standard specimen pot without additives.
- Special blood tests: A sample shall be taken from the antecubital fossa and placed in a standard bottle without additives.
- The patient’s ulcer will be managed using compression bandaging.

4.1.2 Subsequent Assessments

- Reassessments of the patient will take place at weeks 1, 2, 4, 8, 12 and 20.
- These will involve reviewing any significant changes in health.
- The ulcer area will be measured using digital planimetry to chart progress of healing.
- Compression bandaging will be continued as per the requirements of the patient.
- Wound fluid, urine and special blood tests will be repeated as described above.

4.1.3 Week Twenty – Special Arrangements

If the ulcer has healed by week 20 then the patient will have completed the study. If the ulcer has not healed, the patient’s standard of therapy (compression bandaging) will be complemented by the use of an Urgostart® dressing. This product will be used within its licence.

These patients will be followed up to a total of 28 weeks. Assessments as described above will be repeated at weeks 21, 22, 23, 25 and 28. These assessments will be undertaken to detect changes in the microenvironment of the wound in response to a new treatment.

4.1.4 End of the Study

The study is complete once the last patient visit has taken place.

4.2 Standard Operating Procedure for Sample Collection and Preparation

4.2.1 Digital and Manual Planimetry

4.2.1.2 Digital Planimetry

1. Digital camera to take photograph of wound with calibration scale adjacent to wound
2. Computer based calculation of wound size

4.2.1.3 Manual Planimetry

1. Transparent, flexible planimetry grid placed onto wound. Edge of wound traced with permanent marker pen.
2. Calculate area of ulcer and log into clinical data sheet.

4.2.1.1 Urine

1. Mid stream urine sample
2. Transfer to 50ml Falcon tube
3. Aliquot 10 x 1ml samples into 2ml Eppendorf
4. Transported to South Kensington Campus on dry ice
5. Freeze at -80 degrees Celsius for storage

4.2.1.2 Blood

1. Sampled from antecubital fossa avoiding the use of alcohol based skin preparation. Collection into plain blood bottles.21g needle and syringe technique
2. Transfer into 15ml Conical Dome Polypropylene Falcon Tube
3. Stand for 30 minutes to clot on dry ice
4. Centifugation at 2,500g for 10 minutes to obtain serum from supernatant fluid
5. Transfer serum into 10ml Falcon tube
6. Aliquot 0.4ml into 1.5 Eppendorf x 2 (minimum, ideally 3)
7. Freeze at -80 degrees Celsius for storage

4.2.1.3 Leg Ulcer Fluid

a. Occlusive Dressing Technique (6)

1. Ulcerated area covered in Tegaderm dressing
2. 90 minutes with the leg dependent in a seated position
3. Fluid aspirated with hypodermic 21g needle and syringe
4. Fluid transferred into 10ml 15ml Conical Dome Top Polypropylene Falcon tube and kept on dry ice
5. Collected fluid kept on ice and centrifuged within 30 minutes at 13,000g
6. Supernatant filtered using methylcellulose filter (8)
7. Transfer 0.4ml into 1.5ml Eppendorfs
8. Transport to South Kensington Campus on dry ice
9. Flash frozen and stored at -80 degrees Celsius

b. Filter Disc Technique (7)

1. Preprepared 1cm2 absorptive filters from Whatman 54 paper, sterilised in ethanol and oven dried at 60 degrees Celsius. Preweighed in sterile 2ml tubes.

2. Sterile Tegapore mesh placed cut into 4cm2 segments and placed onto ulcer. Ten discs placed on top of mesh.
3. After 90 minutes, disc removed from mesh and placed back into tube.
4. Transferred to South Kensington Campus on dry ice
5. Putative Imperial method – Analyse filter discs directly after separation into aqeous and organic extracts
6. Aqueous extract – mass spectrometry and NMR
7. Organic extract – Mass spec ± examination for eicosanoids / inflammatory molecules

Tarlton method

1. Filter discs reweighed to determine quantity of fluid absorbed and placed into extraction buffer.
2. Four hours of agitated extraction and fluid removed for freezing and stored at -80 degrees.

4.3 Proposed Analyses

These assays would be performed on ulcer fluid, urine and blood samples collected at the initial and each subsequent assessment.

4.3.1 Metabonomics

This technique has been pioneered at Imperial College London and is defined as a technique to broadly measure the "global metabolic metabolic response of living biological systems to biological stimuli or genetic manipulation."(9)

We would seek to obtain a comprehensive metabolic profile of a chronic venous ulcer throughout its cycle of healing or failure to heal. This would be performed using established techniques of nuclear magnetic resonance spectroscopy and mass spectrometry.

Biomarkers of potential significance will be identified from the comprehensive list of metabolites using chemometric and bioinformatics software.

4.3.2 Functional Assays of Angiogenesis

Commercial angiogenesis assays will also be performed to evaluate the angiogenesis stimulating ability of paired wound exudate specimens.

4.3.3 Other First Line Assays

- PAI-1, lactotransferrine, S100A9, Annexine 1, AGEs (advanced glycation end-products)
- Markers of oxidative stress-alantoinuric acid ratio, protein carbonyl content
- Plasminogen activators - uPA, tPA

4.3.4 Second Line Assays

These tests will be performed if the sample volume is adequate.

- Proteolysis – MMP2, MMP9, TIMP1, TIMP2
- Angiogenesis – VEGF, bFGF
- Inflammation – IL1 (α And β), IL6, TNFα
- Fibrosis – TGFβ1
- SERPINB4,
- SERPIN D1,
- Lipocaline 2,

- IFN gamma

Comprehensive metabolic profile will be attained in ulcer fluid at the initial assessment. Changes in this profile over the subsequent assessments will highlight the metabolic response of ulcer healing. Using the chemometric software and multivariate statistical methods, potential biomarkers of ulcer healing will be identified. The changes in metabolic picture will also be correlated with the changes other mediators (PAI-1, lactotransferrine, S100A9, Annexine 1, AGEs, markers of oxidative stress, MMPs, cytokines, growth factors) to elucidate the pathogenesis of chronic venous ulcer formation. Moreover, the effect of Urgostart® dressing on the potential biomarkers of non-healing ulcer will help us to predict the treatment response.

4.4 Data Handling and Record Keeping

4.4.1 Clinical Data

The initial assessment comprising history, examination, routine blood investigations, colour venous duplex result, venous clinical severity score, CEAP classification and assessment of wound status shall be recorded onto a standard document <clinical data document v2>. Subsequent assessments shall be recorded onto <follow up clinical data document v1>.

Clinical data shall be placed into a computerised database and each patient will be given a unique study identifying number. Clinical data documentation will be placed into the study master file.

Unique identifying numbers will be held at Imperial College and at the relevant clinical sites.

The study master file will be kept at the secure vascular clinical research office at Imperial College's Charing Cross Campus.

4.4.2 Sample Labelling and Storage

Samples will be marked with the patient's unique identifying number and a suffix to the sample will be added as per the specimen type.

Blood serum	B
Urine	U
Wound fluid – Occlusive dressing	fX
Wound fluid – Filter disc	fN
Filter disc for analysis	fW

Prior to analysis, all samples will be stored in a secure freezer at the Section of Computational and Systems Medicine, South Kensington Campus Imperial College London at -80 degrees Centrigrade.

4.5 Statistics

Statistical analysis will be performed to analyse the patient recruitments in terms of baseline clinical characteristics. Survival analysis will be used to demonstrate ulcer healing. Biological assays will be analysed using univariate statistical analysis. Metabonomic assay data will be mathematically modelled and analysed using multivariate statistical models.

4.6 Summary of Study Patient Procedures

First Meeting	Introductions, discussion and consent

Initial Assessment – Week 0	Preliminary evaluation – Interview about medical problems, physical examination. Blood, urine and ulcer fluid sampling. Wound dressing.
Week 1,2, 4, 8 and 12	Clinical review Blood, urine and wound ulcer fluid sampling
Week 20	Clinical review Blood, urine and wound ulcer fluid sampling If wound has not healed, apply Urgostart® dressing to wound dressing. If wound has healed – end of study.
Week 21, 22, 23 and 25	Clinical review Blood, urine and wound ulcer fluid sampling Continue dressing with Urgostart®
Week 28	Clinical review Blood, urine and wound ulcer fluid sampling Completion of Urgostart® dressing End of study

5. Adverse Events

5.1 Definitions

Adverse Event (AE): any untoward medical occurrence in a patient or clinical study subject.

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:

Results in death

Is life-threatening – *refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe*

– **Requires hospitalisation, or prolongation of existing inpatients' hospitalisation**
- **Results in persistent or significant disability or incapacity**
- **Is a congenital anomaly or birth defect**

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

5.2 Reporting Procedures

All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator in the first instance.

5.2.1 Non Serious Adverse Events

All such events, whether expected or not, should be recorded.

5.2.2 Serious Adverse Events

An SAE form should be completed and faxed to the Chief Investigator within 24 hours. However, hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.
All SAEs should be reported to the <name of REC> where in the opinion of the Chief Investigator, the event was:
- 'related', ie resulted from the administration of any of the research procedures; and
- 'unexpected', ie an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies. The Chief Investigator must also notify the Sponsor of all SAEs.
Local investigators should report any SAEs as required by their Local Research Ethics Committee, Sponsor and/or Research & Development Office.

6. Regulatory Issues

6.1 Ethical Approval

The Chief Investigator has obtained approval from the xxx Research Ethics Committee. The study must be submitted for Site Specific Assessment (SSA) at each participating NHS Trust. The Chief Investigator will require a copy of the Trust R&D approval letter before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

6.2 Consent

Consent to enter the study must be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent should be obtained. The right of the participant to refuse to participate without giving reasons must be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

6.3 Confidentiality

The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

6.4 Indemnity

Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study.

6.5 Sponsor

Imperial College London will act as the main Sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

6.6 Funding

Laborotoires Urgo are funding this study with an unrestricted academic grant. No specific payments are being made to participants or investigators.

6.7 Audits and Inspections

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

7. Study Management

Day to day management of the study will be co-ordinated through Mr Rahul Velineni, Clinical Research Fellow in Vascular Surgery under the supervision of the Chief Investigator Professor Alun H. Davies.

8. Publication Policy

The aim of this study is to generate results for dissemination to international meetings and peer reviewed articles in scientific journals. Authorship shall be determined by contribution to the various aspects of the study. All authors shall have the ability to review and comment on the manuscript of any work prior to submission. The study shall also form the basis of the higher degree submission of Mr Velineni at Imperial College London.

9. References

1. Fowkes FG, Evans CJ, Lee AJ. Prevalence and risk factors of chronic venous insufficiency. Angiology. 2001 Aug;52 Suppl 1:S5-15. PubMed PMID: 11510598. Epub 2001/08/21. eng.
2. Iglesias CP, Nelson EA, Cullum N, Torgerson DJ, Ven USIc. Economic analysis of VenUS I, a randomized trial of two bandages for treating venous leg ulcers. The British journal of surgery. 2004 Oct;91(10):1300-6. PubMed PMID: 15382101.
3. Gohel MS, Taylor M, Earnshaw JJ, Heather BP, Poskitt KR, Whyman MR. Risk factors for delayed healing and recurrence of chronic venous leg ulcers--an analysis of 1324 legs. European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery. 2005 Jan;29(1):74-7. PubMed PMID: 15570275. Epub 2004/12/01. eng.
4. Rutherford RB, Padberg FT, Jr., Comerota AJ, Kistner RL, Meissner MH, Moneta GL. Venous severity scoring: An adjunct to venous outcome assessment. J Vasc Surg. 2000 Jun;31(6):1307-12. PubMed PMID: 10842165. Epub 2000/06/08. eng.
5. Eklöf B, Rutherford RB, Bergan JJ, Carpentier PH, Gloviczki P, Kistner RL, et al. Revision of the CEAP classification for chronic venous disorders: Consensus statement. Journal of Vascular Surgery. 2004 12//;40(6):1248-52.
6. Gohel MS, Windhaber RAJ, Tarlton JF, Whyman MR, Poskitt KR. The relationship between cytokine concentrations and wound healing in chronic venous ulceration. Journal of Vascular Surgery. 2008 11//;48(5):1272-7.
7. Tarlton JF, Bailey AJ, Crawford E, Jones D, Moore K, Harding KD. Prognostic value of markers of collagen remodeling in venous ulcers. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society. 1999 Sep-Oct;7(5):347-55. PubMed PMID: 10564563. Epub 1999/11/17. eng.
8. De Mattei M, Ongaro A, Magaldi S, Gemmati D, Legnaro A, Palazzo A, et al. Time- and dose-dependent effects of chronic wound fluid on human adult dermal fibroblasts. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]. 2008 Mar;34(3):347-56. PubMed PMID: 18177397. Epub 2008/01/08. eng.
9. Nicholson JK, Lindon JC. Systems biology: Metabonomics. Nature. 2008 10/23/print;455(7216):1054-6.