**STUDY PROTOCOL**

**Near infra-red imaging of the microvasculature in comparison with resin casting of amputated limbs**

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1. Synopsis 3

2. Background 3

3. Objective and Hypothesis 4

4. Contrast Agent 4

5. Preparing the ICG-PULSION solution 6

6. Trial Design 6

7. Equipment 8

8. Safety Reporting 9

9. Ethics 10

10. Financing and Insurance 11

11. Results Dissemination 11

12. References 12

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| 1. Synopsis |

**Study Title:** Near infra-red imaging of the microvasculature in comparison with resin casting of amputated limbs.

**Trial Participants:** Patients referred for amputation of the leg during the trial period.

**Sample Size:** >15

**Planned Trial Duration:** April 2017 to April 2019

**Primary Objective:** To investigate microvenous vessels and their abnormalities using near infrared imaging (NIR) by correlating NIR data with observable venous structures in resin casts of amputated limbs.

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| 2. Background |

Chronic venous insufficiency (CVI) in some patients can lead to varicose veins, pain, venous ulceration, and loss of limb. To date, it is not possible to predict in which patients with CVI the disease will progress. Previously we have shown that venous insufficiency can be prevalent in the microvenous network, even when ultrasound diagnostics show that the Great Saphenous Vein (GSV) has no reflux. We theorize that insufficiency at this microvenous level could indicate the early stages of CVI. Patients deemed to be at risk for CVI could then undertake preventative treatment modalities (exercise, nutrition, compression garments).

Non-invasive imaging modalities that can distinguish abnormal microvenous systems would be a valuable tool in ascertaining patient risk. Near infrared imaging using indocyanine green (ICG) has been used since 1956 in the fields of cardiology, ophthalmology, neurology, and plastics for examining perfusion and identifying perforators. ICG is a dye that is injected intravenously, and that fluoresces under a near-infra red laser camera. The clinician can see the blood flow through blood vessels while the dye is passing through. Previous work by our group in a pilot study suggested that microvenous structures can be imaged, and this study is a continuation of that work.

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| 3. Objective and Hypothesis  |

We hypothesise that these new imaging tools will allow the visualization and quantification of microvenous reflux (i.e., reflux in the tiny skin vessels) which has previously been unseen using standard ultrasound technologies, and that the results of this imaging will correlate with microvenous insufficiencies seen in retrograde resin casting. This may give us further insight into understanding how venous insufficiency leads to skin damage.

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| 4. Contrast Agent |

4.1 Description I include this info about ICG because the SMI protocol did. Is it really necessary to provide this much info on ICG?

**ICG-PULSION**
**Indocyanine Green (ICG) Monosodium Salt Powder for Solution for Injection**
*Spy has a penetration depth of 5 mm.
Half-life in vial is 6 hours following dissolution.
Half-life systemically is ~3 minutes.*

## 4.2 Pharmacology and Pharmokinetics

***Pharmacology:*** The active substance or dye stuff indocyanine green contained in ICG-PULSION is the monosodium salt of 1 –[sulfo-butyl)-3.3-dimethyl-2-{7-[-[(4-sulfo-butyl)-3.3-dimethyl-4.5-benzoindoliny-liden-(2)]-heptatrien(1.3.5)-yl}4.5 benzoindolium iodide. The molecular formula is C43H47N2NO6S2. The molecular weight is 774.97 daltons.

***Pharmokinetics:*** After intravenous injection indocyanine green binds almost completely to globulins, preferentially to α1-lipoproteins, within 1 - 2 seconds. This complete binding within seconds means that uptake by the peripheral tissues, kidney or lung can be practically ruled out and is therefore negligible. In healthy volunteers indocyanine green cannot be detected in either urine or cerebrospinal fluid and it does not cross the placenta.
In patients with normal liver function, elimination of the dye from the blood takes place in the form of a negative exponential function. After 10 minutes only a small fraction of the originally injected volume is detectable in the blood. Indocyanine green is eliminated almost entirely via the liver. It is taken up by the parenchymal cells of the liver, bound by acceptor proteins and eliminated with the bile. The dye undergoes no chemical change on passage through the liver. 15 minutes after passage into the bloodstream indocyanine green is already detectable in the bile, the concentration maximum is reached after about ½ - 2 hours depending on the amount injected. As indocyanine green is not reabsorbed in the intestine there is no enterohepatic circulation.

***Composition of the drug:*** Active ingredients in type and amount: One vial contains 25 mg indocyanine green monosodium salt.

***Other important ingredients (excipients):*** Nitrogen used as an inert gas.

4.3 Contraindications and side effects
***Contraindications:*** As in vitro experiments have shown that indocyanine green displaces bilirubin from its protein binding ICG-PULSION should not be used in premature infants or neonates in whom an exchange transfusion is indicated on account of hyperbilirubinaemia. On account of its iodide content ICG-PULSION should not be used in patients allergic to iodine unless special precautions are taken. If injection of ICG-PULSION was poorly tolerated in the past, it should not be used again.

***Side-effects:*** Anaphylactic or urticarial reactions have also been reported in patients with a history of allergy to iodides. If such reactions occur, treat with appropriate agents, (eg epinephrine {adrenaline}, antihistamines and corticosteroids).

***Possible side effects:***
In very rare cases, after the injection of preparations containing indocyanine green, nausea and hypersensitivity reactions (allergies) may occur. The probability of an allergic reaction occurring seems to be higher in patients with severely disturbed kidney function.
The following symptoms or adverse events may occur: restlessness, hot flushes, nausea, itching, hives (skin rash), faster heart beat, facial redness, swelling of the face (facial oedema), drop in blood pressure, shortness of breath, spasms of the bronchia and larynx, cardiac/circulatory arrest, death. An increase in certain white blood corpuscles may occur in connection with the allergic reaction. In very rare cases, spasm of the coronary vessels has been described. Skin symptoms similar to hives have been described very rarely.

***Major incompatibilities:*** ICG-PULSION must NOT be diluted with solutions containing salts (saline, Ringer's solution etc) as this can lead to precipitation of the dye.

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| 5. Preparing the ICG-PULSION solution |

ICG-PULSION powder is diluted by the addition of 10 mL sterilized water to a pre-prepared 25 ml injection vial, giving a 0.25% solution (2.5 mg/mL).

Dilution: 4 mL of ICG solution in 6 mL of saline (1:2 dilution, 1.25 mg/mL)

1 mL of above solution in 9 mL of saline (1:25 dilution, 0.1 mg/mL)

1 mL of above solution in 9 mL of saline (1:250 dilution, 0.01 mg/mL).

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| 6. Trial Design |

6.1. Trial Participants
Study participants will be chosen from patients at the Dunedin Hospital that are referred for an amputated lower limb.

6.2 Methodology
The study will involve two sessions; initially a session in which patient will be allowed to discuss the project, be familiarized with the protocol, read the information sheet, sign the consent form, and perform the NIR imaging. This session is expected to take 60 minutes.

6.3 Inclusion / Exclusion Criteria

Inclusion criteria:
- Being referred for non-acute amputation of a lower limb.

Exclusion criteria:
- Patient has known hypersensitivity to indocyanine green (ICG)

6.4 Experimental ProtocolEach experimental session will take place in the Otago Vascular Diagnostics Laboratory on the 4th floor of Dunedin Hospital. After being referred for amputation of a lower limb, prospective subjects will be approached to determine if they are interested in taking part in this study, and to make a time for a NIR imaging session prior to their amputation.

***6.4.1 NIR imaging***

During this session, a research assistant will discuss the consent from and protocol, and if participants are willing, will gain written consent. Subjects will be then be instructed to lie supine and bear weight on the contralateral limb with their foot placed firmly on a foot plate. A photograph of the patient’s gaiter region will be taken.

***Placement of venous cannula:*** A trained, certified and experienced Dunedin Hospital staff member will place a standard venous catheter in a suitable foot vein; risk and discomfort for the subject will be minimized as much as possible.

Blood pressure cuffs will be placed on the limb, and the proximal calf pressure will be incrementally increased in 10 mmHg steps from 0 – 100 mmHg.

***Indocyanine-green (ICG) injection:*** Following ICG preparation as described above, 2.5 mL of ICG solution (0.01 mg/mL, 0.025 mg total) will be injected into the catheter, with the initial cuff pressure set at 100 mm Hg.

***Imaging:*** NIR imaging will be undertaken using the SPY system (SPY Novadaq Technologies Inc, Bonita Springs, FL). The emitting laser (805 nm) will be used to fluoresce the ICG contrast, and this will be captured by the NIR camera and recorded and displayed in real time.

***6.4.2 Resin Casting***

The limb will be surgically removed according to Dunedin Public Hospital protocol. Within 1 hour of amputation, the GSV of each limb will be cannulated with a 20 gauge needle at the level of the medial malleolus and flushed with saline. Resin (Batson’s #17 resin, Polysciences Inc. Warrington, Pa) will be injected into the limb. As resin begins to flow from vessels at the proximal end of the limb, these vessels will be identified and ligated. Once the outflow vessels are closed, further resin will be injected until significant resistance is met (less subjective way of doing this?) The limb will be immersed in saline for 12 hours to allow the resin to harden. Tissue will be removed by maceration in 15% sodium hydroxide (3.75 M) at 60oC and the resulting cast will be analysed.

***6.5 Analysis:***

***6.5.1 NIR analysis:***

At the completion of the imaging procedure, captured sequences will be analysed using the SPYQ software (SPY Novadaq Technologies Inc, Bonita Springs, FL). Ingress: the difference in intensity from start to maximum, egress: the difference in intensity from maximum to final, ingress rate: ingress/second, egress rate: egress/second, time to peak intensity, and peak intensity will be recorded.

***6.5.2 Resin Casting analysis:***

As described in Vincent 2011, venous incompetency will be categorised according to how many generations of valves are incompetent. The resin can pass freely into a tributary if the valves in that tributary are incompetent, so with no incompetence, only the GSV is mapped in the resin cast. The GSV and its tributaries are generation 0 (no venous insufficiency). Valves in subsequent tributaries will be assigned a consecutively numbered generation, up to generation 4. It was shown in Vincent 2011 that once the resin passed generations 3-4, the interconnected nature of the microvessels allowed the resin to pass into the small vessel network, regardless of where the insufficient valves were located.

***6.5.3 Statistical Analysis***

Data from the SPYQ software (ingress, egress, ingress rate, egress rate, time to peak intensity, and peak intensity) will be compared with the level of incompetency found in the resin casts (Generations 0-4) using whatever statistical package I have access to.

 Spearman’s rank correlation coefficients will be used to reflect the degree of relationships between variables. Significance will be indicated by two-sided p<0.05.

6.6 End of TrialParticipants will be informed that they can withdraw from the study at any time without giving a reason to do so. The study can also be terminated at the discretion of the Principal Investigator.

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| 7. Equipment |

10 mL syringes
3 way tap
Drip stand
Torniquet
Precision Glide Cannulating Needle (0.5 x 38 mm)
Extension tubing
IV line
Tape
Gloves
Alchohol prep pads (70% isopropyl alcohol)
500mL intravenous infusion (sodium chloride 0.9%)
 **Stuff for macerating amputated limbs (15% NaOH, vats,….)**

Resin

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| 8. Safety Reporting |

All safety events will be classified as follows:

***Adverse Event (AE)***

An AE or adverse experience is:

Any untoward medical occurrence in a patient or clinical investigation in which participants are administered a medicinal product, which does not necessarily have to have a causal relationship with this treatment (the study medication).

***Adverse Reaction (AR)***

All untoward and unintended responses to a medicinal product related to any dose.

A causal relationship between a study medication and the AE must be reasonably possible as judged by either the reporting healthcare professional or the PI.

***Serious Adverse Event or Serious Adverse Reaction***

A serious adverse event or reaction is any untoward medical occurrence that at any dose:

* Results in death,
* Is life-threatening
* Requires inpatient hospitalisation or prolongs existing hospitalisation,
* Results in persistent or significant disability/incapacity, or
* Is a congenital anomaly/birth defect.

***Expected Serious Adverse Events/Reactions***

No serious adverse reactions are expected.

***Suspected Unexpected Serious Adverse Reactions (SUSAR)***

All serious adverse reactions where a causal relationship is identified will be considered to be SUSAR’s.

***Reporting Procedures for All Adverse Events***

All AEs occurring during the study observed by the PI or reported by the participant, whether or not attributed to study medication, will be recorded on the CRF.

The following information will be recorded: patient study number, description of adverse event, date of onset and end date, severity, assessment of relatedness to study medication, other suspected drug or device and action taken. Follow-up information should be provided as necessary.

AEs considered related to the study medication as judged by a medically qualified investigator or the sponsor will be followed until resolution or the event is considered stable. All related AEs that result in a participant’s withdrawal from the study or are present at the end of the study, will be followed up until a satisfactory resolution occurs.

Should a participant be withdrawn from the study due to a serious adverse event then the participant will undergo an end of study assessment and be given appropriate care under medical supervision until symptoms cease or the condition becomes stable.

The relationship of AEs to the study medication will be assessed by a medically qualified investigator.

Any pregnancy occurring during the clinical study and the outcome of the pregnancy will be recorded and followed up for congenital abnormality or birth defect.

***Reporting Procedures for Serious Adverse Events***

All Serious Adverse Events will be reported to the Head of Department within 72 hours of the event for further action.

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| 9. Ethics  |

9.1 Declaration of HelsinkiThe Principal Investigator will ensure this study is conducted in full conformity with the current revision of the Declaration of Helsinki (last amended October 2000, with additional footnotes added 2002 and 2004).

## 9.2 ICH Guidelines for Good Clinical Practice

The PI will ensure that this study is conducted in full conformity with relevant regulations and with the ICH Guidelines for Good Clinical Practice (CPMP/ICH/135/95) July 1996.

## 9.3 Approval

The protocol, consent form and participant information sheet have been submitted to the Health and Disability Ethics Committee, Southern District Health Board Research Office and Ngai Tahu Research Consulation Committee.

## 9.4 Participant Confidentiality

Staff will ensure that the participants’ anonymity is maintained. The participants will be identified only by a participant’s ID number. All documents will be stored securely and only accessible by trial staff and authorised personnel. The study will comply with the Data Protection Act, which requires data to be anonymised as soon as it is practical to do so.

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| 10. Financing and Insurance |

Internal funding through Otago Vascular Diagnostics will cover the salaries of research assistants who will carry out the scans as well as other costs of the research.

In the unlikely event of physical injury arising from participation in this study, patients will be covered by the accident compensation (ACC) legislation with its limitations.

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| 11. Results Dissemination |

The results will be submitted to either local or international peer reviewed scientific journals for publication. The project will also be presented at local, national and international scientific meetings.

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| 12. References |

Vincent JR, Jones GT, Hill GB, van Rij AM. Failure of microvenous valves in small superficial veins is a key to the skin changes of venous insufficiency. Journal of vascular surgery. 2011 Dec 31;54(6):62S-9S.