Imaging the superficial lymphatic system of the lower limb after soft tissue injury.

Malou Catharina van Zanten, *B Health*

School of Medicine, Faculty of Medicine, Nursing and Health Sciences

Department of Surgery, Lymphoedema Clinical Research Unit

Flinders University

Adelaide, South Australia

This thesis was submitted on the 27th of June 2016 as part of the requirements for the degree of Doctor of Philosophy.

DECLARATION

I, Malou van Zanten, certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed.....

Malou van Zanten

Date.....

ABSTRACT

Severe open fractures due to high energy trauma require soft tissue reconstruction with local, regional or free tissue in addition to the fixation of bone injury. Oedema, both within and surrounding the reconstructed site, can present acutely in the post-surgery setting. However, in some patients the swelling fails to resolve and chronic oedema develops. This is lymphoedema, when the lymphatic system is in a state of failure, either due to its inability to regenerate in the wounded area or its inability to handle the increased load imposed in the post-traumatic period. Lymphoedema is the accumulation of fluid in the tissues. As lymphatic failure progresses, lymphoedema worsens, resulting in visible swelling, mobility issues and there can be associated discomfort, heaviness and pain. While lymphoedema is commonly recognised as secondary to breast cancer and its treatment, it can also occur after trauma with extensive soft tissue damage or loss. A review of the literature showed no current best practice protocols are available for traumatic lower limb lymphoedema.

New lymphatic imaging techniques, such as those explored in this thesis which are focused on lymphatic function may however provide an improved understanding of lymphatic failure in these trauma related conditions. With this there is a possibility to identifying the reasons for poor regrowth and inosculation of lymphatic channels, or the effect of increased loads on the existing lymphatic system. The imaging technique which forms the core of this thesis involved the contrast agent Indocyanine Green (ICG), in combination with Near Infra-Red (NIR) imaging system. Its use allows a minimally invasive real-time image of the superficial lymphatic system and provides an indication of its functional status. This means changes to the superficial lymphatic system after soft tissue damage and reconstruction can be assessed.

Severe compound lower limb fractures are associated with extensive soft tissue damage resulting in disruption of lymphatic pathways. The extensive tissue damage often requires transposition of a flap consisting of muscle and/or soft tissue to close any existing defect. These interventions may also influence lymphatic regeneration and pathways. For these reasons an in-depth understanding of an individual's local and general lymphatic architecture is valuable both in the understanding of underlying pathology and for the targeting and tailoring of treatment.

Materials and methods

A custom made near-infrared indocyanine green imaging system was designed and built with the Biomedical Engineering department at Flinders University. This system was tested and validated with *in vitro* and *ex vivo* experiments.

In addition, the system was used in experimentation with genetically modified mice (n=14, *Gata*2 heterozygous) with compromised lymphatic function versus wildtype mice (n=9).

Human research commenced following ethics approval at the Royal Adelaide Hospital. Patients who had a prior reconstructive surgery between 2009 and 2015 as a result of severe lower limb trauma were recruited from an existing database at its department of Plastic and Reconstructive Surgery, Royal Adelaide Hospital. At recruitment basic socio-demographic data was collected and a General Short Health questionnaire (SF-12), and the Lower Extremity Functioning Scale (LEFS) were self-administered. Baseline data at this time was also collected, including that relating to general and site specific fluids using Bio-impedance Spectroscopy (ImpediMed, Queensland) and Tissue Dielectric Constants (TDC) Moisture Meter (Delfin Technologies, Finland) respectively. To measure skin barrier function, Trans-Epidermal water loss was assessed, using a Vapometer (Delfin Technologies, Finland). Leg circumferences at 4 cm intervals were determined using a standard tape measure to determine segmental and overall lower limb volumes. To examine lymphatic function, 0.1ml ICG (25mg/5ml PULSION medical, Germany) was injected intra-dermally into two sites of the dorsum of the foot. In all cases the contra-lateral non injured leg acted as the control. All measurements were repeated at the 12-month follow-up with the patients' consent.

Results

The custom made imaging system showed fluorescence and worked effectively and reliably.

The *Gata2* heterozygous mice (n=11) showed 61% faster uptake of lymph node fluorescence compared to wildtype mice (n=8). Lymphatic pulsation was detected and there was a faster pulsation in the wildtype (n=9, 0.20 beats per second) compared to the *Gata2* heterozygous mice (n=12, 0.15 beats per second).

23 participants were recruited for the clinical study, 94% of the patients were male. The average age at presentation was 47 years with an average duration between the time of accident to the baseline observation of 38 months. Of the patients imaged in this study, 9

had free muscle flaps with a split thickness skin graft, 1 had a free fasciocutaneous flap, 1 full thickness skin graft, 6 a local fasciocutaneous flap and 1 a pedicled gastrocnemius flap. At baseline, the Lower Extremity Functioning Scale (LEFS) of the group was showed participants were on 56% of their normal functioning.

ICG imaging showed none of the free muscle flaps, or the free fasciocuteneous flap, had any functional superficial lymphatic vessels. The local fasciocutanous flaps and the skin graft patients all demonstrated impaired lymphatic vessel function and a dermal backflow pattern similar to that found in chronic lymphoedema. The local fasciocutaneous flaps all demonstrated lymphatic block at their scar tissue edge.

Generally, the uptake of the ICG was delayed in the affected leg compared to their respective control, indicating a reduced lymph transport capacity and flow.

Total water content and extracellular fluid content of the reconstructed leg was significantly higher versus the control leg. Circumference measurements showed a difference between the reconstructed leg and the control but this was not statistically significant.

At the 12 month follow up, 17 out of 23 were re-assessed, and similar results were recorded. Three patients in whom ICG showed superficial dermal back flow common in lymphoedema, were referred to receive further diagnostic testing including lymphoscintigraphy to explore their deeper lymphatic system functioning. This test showed all three to have damage or dysfunction to the deeper lymphatic collectors in the legs

Conclusion

ICG lymphography is a novel imaging technique that may provide information of the superficial lymphatic system pattern in and around the reconstructed area following lower limb trauma, and in small animal experimentation. Severe compound fractures and the associated soft tissue injury can result in significant lymphatic disruption and an increased risk for the development of chronic lymphoedema.

ACKNOWLEDGEMENT

In 2009 I had the good fortune to meet Professor Neil Piller at the International Society of Lymphology meeting in Sydney, Australia, who invited me to "come and do some research," an offer I was only too happy to accept! Neil, your dedication to lymphatic research, your world of knowledge and hospitality has been extraordinary. You are a big inspiration to me, and will continue to be for the next few decades in my professional career. Thank you!

If this were not lucky enough, I was fortunate to have Professor Colin Carati as my supervisor. Thank you, Colin, for everything. I have always admired your down to earth mentality and dedication to science (and cars) and am always surprised at the breadth and scope of your knowledge. I will remember our meetings, where you carefully (and not so carefully) steered me back on track.

Dr Susan Kim showed great patience with me when assisting me with the bio statistical groundwork required for this thesis and my presentations. Many thanks!

It has been a great honour to have Professor Terence Ryan, from the UK, as part of my external supervising panel. The pioneering work he has done and is still doing is incredible, and continues to inspire. Thank you Terence.

Dr James Finkemeyer, without meeting you at the wound clinic at FMC I would not have been able to start clinical research at the Royal Adelaide Hospital. I will always be grateful. Your enthusiasm for my research proposal gave me a great deal of motivation in pursuing my research degree. I hope that in your future career as a plastic surgeon you will continue to show such a keen interest in lymphatics.

Mr Caplash, thank you for your collaboration with your Department of Plastic and Reconstructive Surgery at the RAH, your supervision and access to materials. It has been an absolute privilege working with you.

Thank you to Dr Andrew Campbell-Lloyd for your encouragement and advice in the early stages of research, and invaluable assistance at the start of the human research at the department. Thank you Andrew.

I am very grateful for the assistance of Dr Raakhi Mistry provided me with, and during the first year of clinical research. Thank you Raakhi, your encouragement, interest in the topic of

these neglected lymphatics (and more topics!), your friendship, trust and respect made the entire project feasible.

I am also indebted to the specialists at the Department of Plastic and Reconstructive Surgery: Dr Natasha Forster, Dr Daniel Morritt, research registrar Dr Nick Smith, and nurse Ms Nanindi Fry for assisting with recruitment, materials and ICG. And to Dr Paul van Minnen who kindly answered my questions in Dutch, and offered help when I needed it. Very grateful. Thank you to Dr Ada Lam, in getting the (often last minute!) ICG needed for the research. A big thank you to the lovely ladies that ensured the Department kept running smoothly: Yeng, Anne and Vicky. You have been very supportive and I wish you all the best with the next few research projects to come.

I have been extremely fortunate to have met some of the giants in the field of lymphatics at conferences or as visiting specialists to Flinders University. Professor Leigh Ward visited our research unit in 2014 and assisted me with the SFB7 usage and output. It was a huge eye opener and I am so grateful for your assistance. Having both Dr Vaughan Keeley (Derby, UK) and Professor Margaret Sneddon (Glasgow, UK) visiting Flinders University in 2015 at the same time was a great way of receiving all your advice about pursuing a career in science/academia and clinical lymphatic related research. Thank you so much.

Thank you to Dr Michael Bernas, who worked with me and my custom made camera during his visit to Flinders University. I hope you think of me as one of the people who changed your opinion about ICG and lymphatic imaging. Professor Hiroo Suami, your presentation at the ALA conference in Cairns in 2012 gave me the inspiration to use ICG imaging. Your knowledge of this technology is extensive! I am forever grateful for your help and support, from way back at the beginning of the project, all the way up to the very day I submitted this thesis.

I am very grateful to Professor Natasha Harvey. I am extremely interested in all the work you have done and continue to do in your lab. It was a real privilege to work with you on mice ICG imaging. I hope this will be a method for imaging in the near future. Thank you to Jan Kazenwadel, who took me by the hand to show me molecular biology and small animal research! It was a great experience learning how to shave and "Nair" a mouse!

A special thank you to everyone at the FMC / FUSA Biomedical Engineering Department; In particular, the biomedical legends Dr Olivia Lockwood, Dr Mark McEwen, Dr Lynne Langman, Mr Jack Beesley, Dr Nigel Kelly and Mr Simon Huntington. Thank you to all who

brought my wish to use ICG imaging in my research to fruition. I have made a big effort to make the invaluable contributions of Biomedical Engineering known to all, and I hope to continue working with a dedicated biomedical department like yours!

During the ICG experiment phase, I have been able to source scavenge porcine material free of charge from SAHMRI Gilles Plains. Big thank you to Dr Tim Kuchel and Dr Paul Herde, the Animal House Flinders University, Roxanne Collingwood and Lisa Owen for their assistance.

All my wonderful participants in 2014 and 2015 thank you for dedicating your time and leg(s) for science.

Thank you to the talented David Heinrich for the images in the introduction (Chapter 1) and results (Chapter 5).

I am extremely grateful to Simon Rushworth from SDR scientific for his assistance in getting the Hamamatsu PDE TGA approved in Australia and allowing the RAH plastics department to be the first to use it.

Thank you to all my lymphy friends for your moral support and proofreading stuff during my PhD, overseas, interstate and locally. Toni Piller, Jan Douglass, Sharon Tilley, Marielle Esplin, Beth Kean, Edward Mitchell, Jennifer Byrne, Joyce Bosman, Wouter Hoelen, Monique Bareham (and her Id.)

Thank you so much to all my dear friends in “Straya” for feeding me, PhD procrastination activities such as climbing in SA and VIC, skating (and competing at Nationals!), and helping me let my hair down at all those SA music festivals!

A special thank you to the Lymphoedema Support Group of South Australia for financial support for me to attend an international conference and your dedication to lymphoedema awareness. Monique, Karen, Ruth, Colomba, and Graham you are so important and doing a terrific job!

To all the women of Soroptimist International Adelaide. Thank you for your financial and moral support in 2012 to allow me to go to the ILF conference in Montpellier, and thank you in particular to Marrion Saunders and Pam Lacey for staying in touch every year since.

Thank you to Flinders Clinical and Molecular Medicine Cluster, Research Project Seed Funding or Innovative Technology grant (2012), that allowed us to purchase materials needed for the custom made camera.

Thank you to the Flinders Foundation and Flinders Medical Centre volunteers for conference funding in 2012, 2013 and 2014.

To all my friends and family back 'home' in the Netherlands; thank you so much for all your moral support, visits to South Aussie, endless skype sessions and old skool postcards full of encouragement. My second family; Jannes, Mannie, Stefan and Leontien, thank you!

Last but not least, I want to thank my parents who I am forever grateful for their support and encouragement and “aim for the moon” mentality. My best, beautiful sister in the world Sarah, and Jork, Lois and little Loek, I miss you every day! My genius brother Dr Thomas van Zanten (assisted with Chapter 3) and my “new” sister Dr Ksenia I’ll come and visit you in India very soon. Thank you all for your 'you can do it' encouragement!

Finally, to my partner in crime for the last twelve years, Erwin, I could not have done any of this without you.

Contents

List of Figures

List of Tables

Abbreviations

ALA	Australasian Lymphology Association
AIHW	Australian institute of health and welfare
ATR	Australian trauma registry
AusTQIP	Australian Trauma Quality Improvement Program
AVI	Audio video interleave
BEC	Blood endothelial cell
BIS	Bioimpedance spectroscopy
BMI	Body Mass Index
CCD	Charge-coupled device
CT	Computed tomography
CTN	Clinical Trial Notification (scheme)
DEXA (DXA)	Dual emission X-ray absorptiometry
ECF	Extracellular fluid
FDA	Food and drug administration-United States of America
FFM	Fat-free mass
FM	Fat mass
GMO	Genetically modified organism
ICF	Intracellular fluid
ICG	Indocyanine green
IMP	ImpediMed
IR	Infrared
IQR	Inter quartile range
khz	Kilohertz
LEC	Lymphatic endothelial cell
LED	Light-emitting diode
LEFS	Lower Extremity Functional Scale
LTW	Local tissue water
LVAP	Lymphatic Vessel Analysis Protocol
MBA	Motorbike accident
MCS	Mental Health Component Summary (score)
mG	Milligram
mL	Millilitre
MRL	Magnetic resonance lymphangiography

MVA	Motor vehicle accident
mmHg	Millimetre of mercury
NIR	Near-infrared
nm	Nanometre
PCS	Physical Health Component Summary (score)
SA	South Australia
SAHMRI	South Australian Health and Medical Research Institute
SF-12	General Health Short Survey Form
SF-36	General Health Questionnaire
SLN	Sentinel lymph node
SPECT	Single photon emission tomography
RAH	Royal Adelaide Hospital
TBW	Total body water
TDC	Tissue dielectric constant
TEWL	Trans-epidermal water loss
TGA	Therapeutic Goods Administration (Australia)
VAC	Vacuum-assisted closure
VEGF	Vascular endothelial growth factor

CHAPTER 1
Introduction to thesis and literature review

This Chapter will present statement of the research problem, the objective of the research and potential contribution to improving patient health outcome and to the scientific community. This is followed by an introduction to the thesis and a comprehensive literature review concerning the lower limb trauma patient demographics in Australia, the lymphatic system, reconstructive surgery, and flap physiology. The literature review in this Chapter is based on the peer reviewed publication: Van Zanten et al. "A review of severe lower limb trauma with extensive soft tissue loss and subsequent reconstructive surgery: Its impact on the lymphatic system". Wound Practice and Research 21(2)- 2013.

1.1 Statement of research problem

This thesis covers the area of lymphatic imaging in patients who have had severe lower limb trauma with subsequent soft tissue reconstruction. This research contributes to the knowledge of lymphatic imaging, and to the knowledge of the recovery process of the lymphatic system in response to lower limb injury with the innovation of utilising a custom designed and built lymphatic imaging camera.

Acknowledgement and awareness of the influence of extensive soft tissue and bone damage in the lower limb on the lymphatic system is lacking in the published literature and in clinical practice, perhaps because the lymphatic system has been hard to visualise, much less study. New lymphatic imaging techniques such as Indocyanine Green (ICG) with Near-Infrared imaging allow better investigation of the lymphatic system and its functionality within the lower limb after reconstruction.

I believe the use of ICG and a custom made near-Infrared camera system has been an appropriate and cost effective method to assess the lymphatic vessel functioning in patients

who have had severe lower leg trauma with associated soft tissue injury. With a longitudinal follow-up, information of lymphatic regeneration in response to injury over time and any information of the contribution of the imported flap tissue to lymphatic function and limb recovery was explored.

1.2 Objective of research

- To assess how high impact soft tissue trauma affects the lymphatic system, using superficial lymphatic imaging and fluid measurement tools.
- To explore if a correlation exists between functional subjective outcomes and objective measurements of the lower limb post trauma.
- To describe, using imaging, any deficit of the lymphatic system following lower limb trauma and the mechanisms and timeline for its repair and the impact of soft tissue coverage on it.
- To better understand the impact of the various soft tissue reconstruction techniques on the lymphatic system and on lymphoedema severity, development and remediation.
- To determine the role of soft tissue reconstruction and key parameters in restoring lymphatic functionality and thus tissue and wound health.

1.3 Contribution to the scientific community

This research contributes to the knowledge of lymphatic imaging, and to the knowledge of the recovery process of the lymphatic system in response to lower limb injury. It also highlights the design and build of a custom lymphatic imaging camera.

1.4 Introduction to thesis

This thesis consists of two parts; a technical part that describes lymphatic imaging techniques followed by experimental validation and quantification approaches. The second part consists of the human research that took place at the Royal Adelaide Hospital in collaboration with the Department of Plastic and Reconstructive Surgery.

1.4.1 First part of the thesis:

An introduction into the study population, the lymphatic system, and a comprehensive literature review can be found in *Chapter 1: Introduction to thesis and literature review*. The aim of this chapter is to identify the gap in the literature regarding lymphatic involvement in wounds and bone healing. General anatomical structure of the lymphatics is discussed as well as a brief introduction into lymphatic system imaging.

Lymphatic imaging will be further explored in depth in *Chapter 2: Imaging the lymphatic system*. This chapter describes the history of lymphatic imaging, the techniques of the past and present as well as elaborating on Indocyanine Green (ICG) and Near-Infrared (NIR) imaging specifically along with details such as testing and validity results of the custom made NIR imaging system.

In *Chapter 3: Real-time lymphatic imaging in mice* there is explanation of the use of the ICG imaging system in small animal research. This pilot study of a lymphatic mouse model versus wildtype mice explores the potentials of ICG imaging quantification.

1.4.2 Second part of the thesis:

Chapter 4: Materials and Methods describes the materials used for the clinical human research part of the thesis including their validity and reliability. The method of measurement, and analysis is explained. This is in preparation for the clinical results that are

described in *Chapter 5: Human research results*. This chapter includes all results of the measurements obtained and further analysis of images.

The thesis concludes with *Chapter 6: Discussion and conclusion* where the thesis is discussed, major findings are collated, and final conclusions are drawn.

1.5 Background of trauma injury: A significant public health problem

In 2011, 26 designated Australian trauma centres and 4 state-based trauma registries joined together to develop the Australian Trauma Quality Improvement Program (AusTQIP) underpinned by an Australian Trauma Registry (ATR) (1). By joining these centres, the collection and processing of the available data will ensure a better outcome for trauma patient management.

South Australia (SA) has 7.2% (1.6 million) of all Australian residents with a population density of ~2 people per square kilometre (2, 3). SA currently has 22 private and public hospitals in the metropolitan region and 39 regional hospitals. The Royal Adelaide Hospital (RAH) is the largest level one adult trauma centre in the state, with over 51,000 patients seen in the Emergency Department every year . In addition, metropolitan SA also has the Flinders Medical Centre and Women and Children's Hospital which also holds a State Trauma Registry and are collaborators in the AusTQIP as described above (1).

Severe trauma or high impact trauma often results in multiple body regions being affected. This type of trauma is often related to a motorised vehicle collision, motorbike, bicycle, and falls from a height or high impact work related accidents. The highest frequency of these is in the population below the age of 45 years (1, 4).

Moderate and especially severe high impact traumatic injury is a main cause for permanent disability, morbidity and mortality and is recognised as a major ongoing public health

problem within Australia (1). The National Trauma Research Institute records show that traumatic injury accounts for 7% of the total burden of disease in Australia (1). The impact of injury is not only evident in the individuals physical functioning and psychological morbidity but also in its effects on society and overall health care cost (1, 5). In 2012, the estimated annual costs are estimated to be AUS $18 billion (1).

1.6 Lower limb trauma demographics

The most common injuries leading to hospitalisation with the young and middle age population are mostly related to transport accidents and falls accounting for 36% of all hospitalisations in Australia (6, 7). The Australian Institute of Health and Welfare (AIWH) series report of the financial year 2009 – 2010 indicated the overall main cause of injury requiring hospitalisation is falls (38%, n=161,147) followed by unintentional transport-related incidents (13%, n=54,110) (8). This is the opposite to a report of the Australian Trauma registry (2010-2012) focused on major trauma hospitalisation where it is stated that mechanism of injury in major trauma (n= 16,644) is mostly transport related (52.4%, n= 10,300), followed by falls (31.2%, n= 6,121) (9). Of the data available on affected body region in the Trauma Registry report (n= 20,435) lower limb injuries accounted for 34% (n= 7023). With all reports agreeing on more men than women are involved in transport injuries (8-10).

Research conducted at the Royal Adelaide Hospital Emergency Department from 2000 to 2003 showed that severe pelvic and lower limb injuries occurred in 21% (59) of 282 trauma cases. Most were related to falls from a >2 meter height either at work or home (3). As indicated above, traumatic injuries can result in a reduced quality of life, impaired functional outcomes and a large and continuing associated cost of medical care (11, 12).

It is extremely difficult, if not impossible, to obtain a detailed epidemiology specifically of trauma related lower limb fractures or open and/or closed long bone fractures due to the

complexity and involvement of injury to other structures, not to mention the national and international hospital coding differences and discrepancies (7, 13).

1.7 Lower limb trauma recovery

Severe lower limb open fractures including ruptured skin and soft tissue are the most common injuries in the Emergency Department (ED) that require hospitalisation and immediate surgical attention (6, 14). The lower extremity contains vital long bones that are vulnerable to injury in high-energy trauma. In particular, the anatomically superficial location of the tibia means that it is less protected by fatty subcutaneous tissue or muscle and is often fractured with associated soft tissue loss.

Salvage of the lower limb is dependent on the nature of the bone injury, gross tissue contamination leading to high infection risk, and the extent and nature of soft tissue damage. After initial trauma management, patients require orthopaedic surgical intervention to stabilise the fracture(s). The remaining wound or soft tissue loss or damage is restored based on careful planning by plastic surgical specialists. The type of soft tissue reconstruction is selected based on any existing co-morbidities, the extent and nature of injury and is a crucial part of limb salvage.

1.8 The lymphatic system; an overview

The lymphatic system is indispensable; a non-functioning or non-existing lymphatic system is not compatible with life, and yet we still have much to learn about this system. The blood vascular system has been studied reportedly back in the sixth century BC, whereas the lymphatic system was not discovered until centuries later. Remarkable as described by Scallan et al. (2010) it is not necessarily the late discovery of the lymphatic system but more the misperceptions encountered during and even since its discovery (15). Misperceptions such as stating that the lymphatic system was merely a passive system for return of excess

fluid, poor understanding of lymph formation, and poor understanding of the detailed lymphatic systems involvement in healthy fluid homeostasis (16, 17). Extensive research in mice and zebra fish embryos in the recent years have established important knowledge regarding the embryonic development of this system. The lymphatic system sprouts from the cardinal vein where the lymphatic endothelial cells (LEC) separate themselves from the blood endothelial cells (BEC). It then goes on to develop a superficial network, connecting vessels or so called pre-collectors, and a deeper system with larger vessels; the collectors (18). This development occurs approximately between week 6 and 7 of human embryonic development, 4 weeks after the establishment of the blood circulation (16).

The lymphatic system is vital for tissue and cell homeostasis, and relies on the balance between the entry of lymphatic fluid from tissue and the ability of the system to transfer that fluid centrally. The lymphatic system is responsible for transporting proteins, fluid, fatty acids, and macromolecules (19). It also actively participates in immune regulation triggered by response to pathogenic and immunogenic stimuli by the peripheral lymphatics (20).

Unlike the vascular system, the lymphatic system is not a closed circulatory system. This means lymph can be transported from distal to proximal areas, and also from the deep system to the superficial system or vice versa. A vital characteristic of the lymph capillaries is that they are highly permeable in order to gather up interstitial fluid and its contents (21, 22). This is transferred to major lymph collectors that pump fluid centrally. These are often situated within the adventitia of large arteries and veins, where lymphatic flow is assisted by arterial pulsatile flow, and tissue variations (23). As lymphatic fluid is slowly transported through the body it enters lymph nodes. Each lymph node acts as a filter, which facilitates immune cell trafficking and provides a key role in activating immunologic responses (23, 24).

The lymphatic capillaries and collecting vessels form a delicate system, and its interdependence with the vascular system makes it vulnerable to damage caused by surgery, mechanical trauma or infections (24).

1.9 Lymphatic microcirculation

The lymphatic vessels structure, specifically of the superficial system, provides insights to its function (19). Microcirculatory lymphatic plexus is present in the superficial layers of the skin, which acts in conjunction with the vascular system to maintain tissue fluid homeostasis. The lymph capillaries, with their distinct single endothelial cell layer and button like junctions, are highly permeable capillaries which take part in the exchange of fluid, cells and macromolecules (19). Much of the formed lymphatic fluid is dispersed superficially to the deep fascia, but some is further transported sub-fascially to the deeper lymphatic system. Lymphatic fluid drains from the peripheries to the abdomen where it travels along tributaries to the thoracic duct and is in turn introduced back into the venous system (19, 22). This process of filtration and reabsorption ensures the balance of the interstitial pressure, where balance of the fluid volume and its contents are maintained (23).

1.10 Impaired lymphatic function

When filtration of the blood capillary system exceeds the reabsorption capacity of the lymphatic system, interstitial homeostasis is disturbed. This is initially characterised by localised tissue oedema. Local tissue oedema can spread as nearby systems are overloaded and can cause a segment, or even the whole limb, to swell (21). Additionally, the generally protein-rich fluid accumulation in the tissues can induce inflammatory reactions due to increased concentration of proteins, such as cytokines, in the lymph and surroundings. This leads to fibrosis, impaired immune responses and reduced wound healing capacity (19). Delivery of crucial nutrients to cells is reduced or completely prevented, causing an overall

patho-histological picture of chronic inflammation (21). Contrary to previous theories, recent research suggests that the lymphatic system is responsible for 100% of the reabsorption process, and is therefore central to local control of superficial fluid homeostasis (17, 25). Research has also emerged, revealing the relationship between fat tissue and lymphatic stasis. Histologically an accumulation of inflammatory cells together with fibrosis, dilated lymph vessels and proliferation of adipose tissue is observed (26). This has raised some questions regarding the influence of adipogenesis on lymphangiogenesis and vice versa.

1.11 Lymphoedema

Lymphoedema is a progressive chronic condition caused by the accumulation of fluid with a higher content of protein in the body tissues. Lymphoedema can be hereditary as a primary condition, or acquired secondarily due to damage or disruption of lymphatic vessels; most commonly in western countries lymphoedema is considered to be due to malignancies and their treatment (21, 24). Lymphoedema can also occur following trauma, to which injuries in the superficial or deep lymphatic systems are sustained (27). In high-energy impact trauma involving injuries to soft tissue in combination with bone, the lymphatic system can be profoundly damaged and efforts at reconstruction are often ignorant to, or ineffective at, re-establishing lymphatic function. Soft tissue reconstruction often involves importing tissue, either locally or from a distant site, to cover exposed bony injury and re- establish the integrity of skin coverage (14). Although the vascularity of such tissue is ensured by the surgeon, there is little capacity to address lymphatic function of such tissue – and such, these patients are at risk of developing lymphoedema in the recovery period and long term.

If the load on the compromised lymphatic system is higher than its transport capacity then the swelling will increase and result in regional or whole limb discomfort, heaviness or pain. The inevitable scar tissue that develops as a result of injury and subsequent surgery prevents the development of lympho-lymphatic anastomosis. Scar tissue across lymph collectors may

present blockage to lymphatic drainage pathways and subsequently cause a reduction in lymph transport (21, 22). There is a delicate balance, which if disrupted, will result in the onset of lymphoedema.

In Australia, lymphoedema secondary to cancer is most often reported with an estimation of at least 20% of those who are treated for melanoma, prostate, breast or gynaecological cancer (29). By best estimates, one in thirty people are affected by lymphoedema worldwide (30). Firm incidence or prevalence figures are almost impossible to accurately determine. Current estimates likely understate the prevalence of lymphoedema given that; the early stages of lymphoedema frequently go unrecognised or are misdiagnosed. Also, there remains little conformity on specific diagnostic criteria making inter-study comparisons difficult (29, 31, 32). Risk factors recently associated with lymphoedema development include obesity. In people with a body mass index (BMI) of more than $30kg/m^2$, which increases the likelihood to develop lymphoedema post-operatively by nearly 4 fold (31). This is concerning given the public health issue of increased obesity in the general western population including Australia which reported an overweight or obese population of 60% in the latest national survey (2011-2012) (33).

Late stage chronic lymphoedema is characterised by an increase of local adipocyte size, adipocyte numbers and local tissue induration (21). Since the local and often general, specific and nonspecific defence systems are compromised when the lymphatic system fails, these patients remain prone to recurrent infections and inflammation (24). Patients affected by lymphoedema not only carry the burden of physical consequences; psychosocial consequences such as distress, depression and reduced body image have also been evident in previous studies (21, 29, 31).

Irrespective of the cause of the lymphoedema or the region affected, generally the physical, social and psychological issues are similar.

Lymphoedema is now identified in the International Classification of Functioning, Disability and Health (ICF). ICF is part of a World Health Organisation (WHO) framework to measure and define health and disability through a specific classification system using descriptions and coding (35). Descriptions are culturally adapted but the coding is identical worldwide. Therefore, ICF creates a common language understood by patients, all health care providers, policy makers and researchers. With this important development, it is evident that people with lymphoedema are affected in all four components of the ICF. These components are; body functions, body structures, activities and participation, and environmental factors(34). With consistent information as provided by ICF coding it will be easier to recognise the severity of debilitation of lymphoedema and the restrictions in active participation in the patients' daily lives. The ICF study is ongoing to eventually develop a core set or short form that will be easily applicable by any health professional for daily clinical use.

1.12 Lower limb compound fractures classification and complications

The role of non-cancer related trauma in lymphoedema is less well defined than lymphoedema as a sequelae of cancer treatment. However, it is an important factor in determining the long-term impact on function and mobility following trauma.

Blunt injuries may be caused by acceleration, deceleration, compression or shearing injuries resulting in various tissue components being crushed, macerated or devitalised entirely. In Australia the majority of trauma are related to motor-vehicle collision and falls (4). These injuries seem to be divided between, older people who generally have a higher incidence of falls whereas the young and the middle aged have more transport-related fractures (6, 10). Such injuries result in a reduced quality of life, impaired functional outcome and large associated cost of medical care (11, 12).

The lower extremity contains long weight bearing bones that are vulnerable to injury in high energy trauma. In particular, the anatomically superficial location of the tibia means that it is less protected by fatty subcutaneous tissue or muscle and is often fractured with associated soft tissue loss. Tibial shaft fractures are the most common long bone fractures in the body, and due to its close connection by ligaments the fibula is also often involved. A few crucial factors such as; initial displacement (the line of the fracture), comminution (splintered / crushed parts), and extend of soft tissue injury (open or compound fracture) determine the clinical outcome (36). An open fracture should be managed with extreme care and is a surgical emergency due to damage of not only the bone but also soft tissue, blood and lymphatic vessels at the micro and macro levels (37). Open fractures are likely to be linked with poor healing and superficial infection as well as infection of the deeper (sub-fascial) and bone areas (36, 38). Fractures are the most common type of hospitalised injury and treatment requires immediate irrigation and debridement (28). Severe compound fractures have been categorised into the Gustilo classification (I to III A, B, and C) since 1976 (38). Gustilo type I and II fractures are clean and the relatively small soft tissue wounds can often be closed primarily. Gustilo type III fractures involve significant amounts of soft tissue loss. Types III are further subclassified into types A, B and C according to periosteal stripping and extend of damage to vascularity. Complications such as infection and non-union specifically following Gustilo III B fracture reconstruction have been well documented (39). However, the extent of damage to the lymphatic system and subsequent risk of recurrent infections and chronic lymphoedema remains relatively unstudied.

Salvage of the lower limb is dependent on the nature of the bony injury, gross contamination leading to infection, the extent and nature of soft tissue damage. Even after reconstruction has been achieved an ongoing risk for deep and superficial infection is associated with open fractures and can result in serious long term complications (40, 41). The average healing time for displaced, open, or comminuted fractures is 4 to 6 months (36).

1.13 Soft tissue damage and the lymphatic response

Every wound is at risk of infection irrespective of cause, size, anatomical location and management (42). When the lymphatic system is damaged and loses its immunological functionality, the risk is greatly increased (43). The frequency of wound infection depends on the type of trauma, location of the wound, presence of contamination and adequate initial debridement and decontamination followed by suitably timed reconstruction (30). Open wounds with extensive soft tissue loss have a poor healing prognosis unless properly reconstructed.

The process of soft tissue repair is inseparably linked to the restoration of lymphatic function. An open wound associated with a compound fracture results in a complex and acute process of repair. Acute wounds undergo an initial phase of coagulation followed by a complex inflammation cascade mediated by lymphocytes, macrophages and granulocytes (42). All of which most often facilitate the healing and normalisation process, but can be sub-optimal if the lymphatic system is dysfunctional. Although lymphatic capillaries have a strong ability to regrow in healthy tissue, in damaged tissue this is more challenging (44). Soft tissue trauma will disrupt microvasculature and its repair processes must come into play. This means an increase in plasma filtration and lymph accumulation in the dermis along with dilated and thus sometimes dysfunctional collecting lymphatics (24).

Information regarding regeneration of lymph capillaries in human wound healing and their importance is scarce. Nogami et al. (2009) visualised lymphatic regeneration in rats, demonstrating the regrowth of lymphatic capillaries from the healthy tissue edges of the wounds. This is different from blood vascular regeneration that rapidly forms in the granulation phase of acute wound healing (45). In general, open wounds demonstrate significant delay in lymphatic channel regeneration in comparison with arterial and venous angiogenesis. In the soft tissue surrounding wounds there is the accumulation of waste

products in the tissues which mechanically compromise lymph capillaries and micro-vascular flow, thereby increasing capillary permeability and venous after load (30). This process adds considerable burden to an already damaged lymphatic system. Essentially overloading an under- functioning system leading to increased risk of failure, infection and delayed tissue healing. The load is increased but transport capacity has been reduced so there is a high chance of failure, with infection and poor tissue healing being the major negative outcomes.

1.14 Flap reconstruction and general physiology

The treatment of compound fractures with extensive soft tissue loss depends on the overall presentation of the extent of bone, soft tissue and vascular injury and requires a multidisciplinary approach (41). Therefore, there is no standard practice that can be applied to all patients. However, treatment such as immediate debridement of contaminated and devitalised tissue followed by reconstruction with either skin grafting in minor wounds or more complex reconstructions such as muscle flaps for extensive injuries. Reconstructions have been introduced in medicine and surgery for over 300 years. Although the first few reported procedures of vascular ligature were reported in the mid-1500, stronger publications regarding lower limb reconstructions arose from the early 1960 (46). Since then reconstructive surgery is continuously revised and adapted in complicated cases. The so called reconstructive ladder has been developed to describes the reconstructive management based on complexity of the soft tissue defect (48). The reconstructive ladder starts with minor procedure such as skin grafts that are commonly used over exposed muscles, fascia or subcutaneous tissues. Local and regional flaps are harvested near the wound area, as it contains a similar blood supply. These are used for small to moderate wounds (Figure 1-1 a-b). A muscle or a fasciocutaneous flap (Figure 1-1 c-d) can be applied in various ways including being rotated, transposed or transported from another part of the body into the

wound (14). The benefit of the muscle and fasciocutaneous flap is the local blood supply which is essential for healing. Free flap reconstruction is the most complex intervention which is used to close large defects that are not otherwise graftable or amendable to local reconstruction. This type of flap contains tissue harvested from another area of the body containing an artery and vein which is re-attached in the defect site (14). Most commonly for the lower limbs are the anterolateral thigh flap (ALT), the latissimus dorsi muscle flap or gracilis muscle flap. The re-attachment from vein to vein and artery to artery insures tissue vascularisation whilst creating an ideal tissue coverage of a healing fracture.

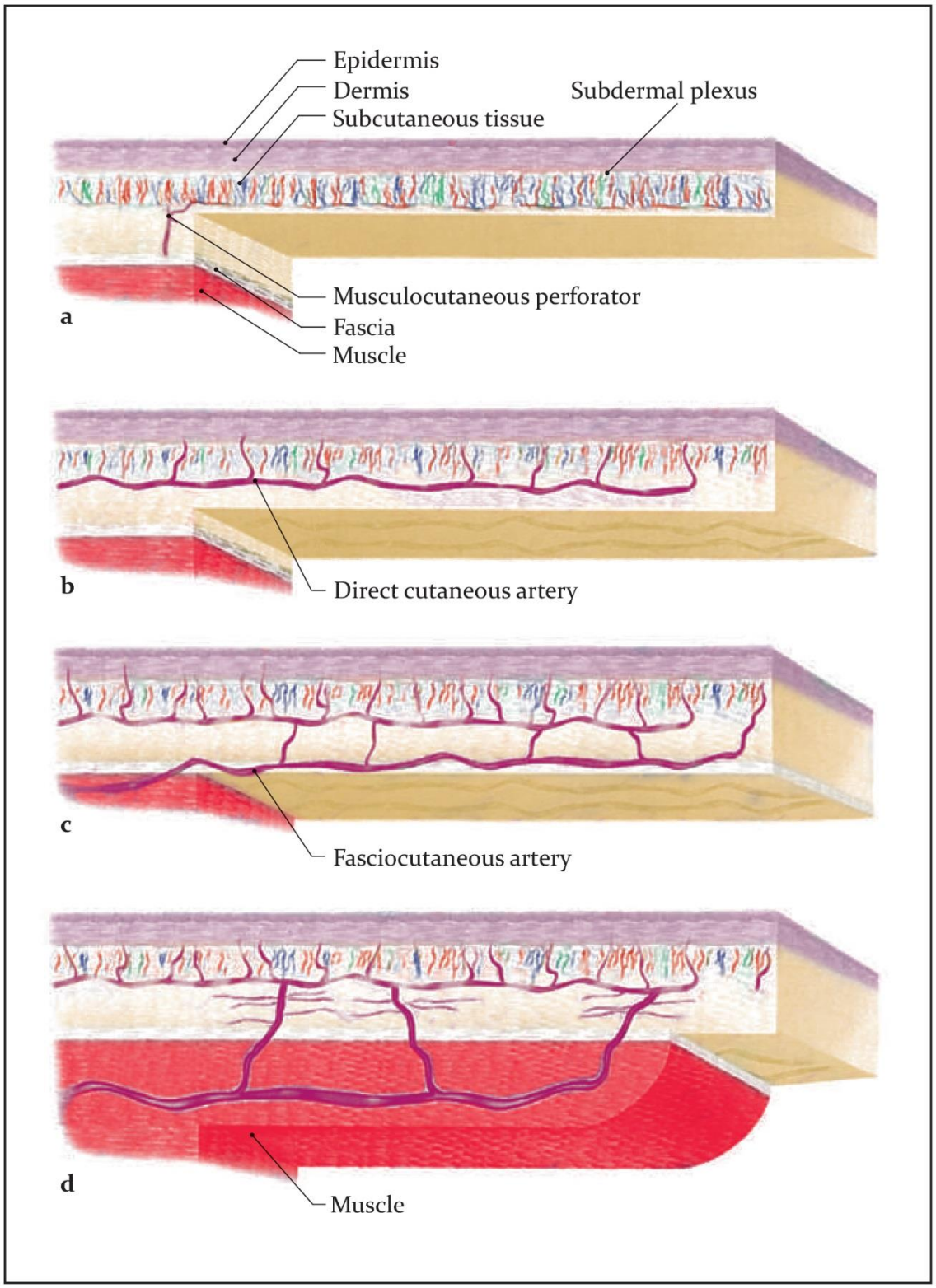

Figure 1-1: Different layers for reconstructive consideration and arterial supply. Figure adjusted and based on Figure "classification of skin flaps" published by Gaboriau et al. (2001)(48).

Despite being technically demanding, the success rate for free flap reconstructions has been estimated as high as 91% (47). Survival is dependent on revascularisation, and is determined by monitoring the continuous arterial flow and venous outflow (47). Venous outflow obstruction is seen as the main cause of free flap failure (49).

Survival of a reconstruction with a free muscle or free fasciocuteneous flap is based on adequate tissue perfusion by its vascular pedicle. Neovascularisation may be triggered by ischemia tissue in the wound. Complications in venous sections of the flap, such as thrombus development or kinking of the vein, induce toxic products but is also strong stimuli to induce neovascularisation (50).

A study in which the muscle dimensions were measured over a range of pre and postoperative time points with ultrasound, CT and water displacement showed oedema in free microvascular flaps to be common but poorly recognised in research studies (51). Their results show an increase of oedema in muscle flaps at the immediate post-operative period and slightly less in 3 months and 9 months' post-operative. The reason flaps become so oedematous immediately post-surgery is unclear, but could be attributed to the increase in blood flow into a more leaky vascular bed, and decreased lymphatic drainage due to surgical trauma. Regrowth of lymphatics in muscle flaps has not been studied and there is a need for further experimental and clinical studies in this area (51).

1.15 Reconstruction and the lymphatic system

It is well accepted that lymphatic vessels contribute to healthy tissue, but the role of the lymphatic microcirculation and subsequent lymphatic regeneration in flap reconstruction has not been researched thoroughly.

After reconstruction, there is an ongoing risk of deep and superficial infection which is commonly associated with open fractures and can result in serious complications such as flap

failure (necrosis), non-union, and chronic osteomyelitis (40, 41). The highest risk for infection occurs prior to full healing of the fracture. Hospital costs are high and there is a significantly reduced quality of life in cases of non- or delayed bone union (52). These events impose further burden on the health care system and an increased level of disability for the "at risk" patients. The overall post trauma patient outcome is, and should always be, aimed at regaining bone union and full weight bearing as well as a pain free and functional lower limb (53).

Oedema in the free flap post-surgery has been identified as a result of impairment in lymph transport (54). Khazanchi et al (1997) explored lymph transport in free flap reconstruction sites using lymphoscintigraphy. Their eight participants showed lymphatic activity in the free flap early after surgery (nine days) (55). This was supported by a study of Slavin et al. (1996) where they performed lymphoscintigraphy 13 days' post-surgery, and found indications of advanced regeneration probably starting as early as eight days (54). Although both studies mentioned fast uptake of the radio colloid tracer and therefore excellent lymph drainage, they did not focus on any abnormal lymph node size (enlargement) or lymph collector dilation.

Persistent oedema has been observed long after the fracture and wound have healed, suggesting that lymphoedema is present (56). Often there is a late diagnosis of this chronic condition, as the clinical symptoms of lymphoedema have progressed or increased or caused immobility (57). Thus, the factors that contribute to post-trauma lymphoedema should be explored so that early intervention can be initiated (58).

Szczęsny et al. (2002 and 2003) explored post-traumatic oedema and found enlargement of lymph nodes in all 21 patients with post-traumatic oedema (59, 60). Lymphoscintigraphy often confirms dilation of major distal lymph collectors in the entire lower extremity and

associated decrease of lymph flow. This adds to the problem of wound repair, healing and infection.

Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) studies show reduction in muscle and an increase in fat in patients with a mean of 30 months follow up. The visible adipose fatty tissue penetrates the muscle fibres where at the same time this process correlates with muscle atrophy (61). Neovascularisation seems to be continuous but there is no mention of lymphatic vessel involvement. However, we do know from recent research that there is a direct link between adipose fatty tissue and lymphatic dysfunction. This is an area worth investigating in more detail.

1.16 Patients immediate and long term outcome

Unfortunately, research questionnaires and research tools aimed to assess the functional outcomes of patients following open lower limb fractures do not include or acknowledge lymphatic failure and thus lymphoedema as a parameter of interest (12, 57). This failure to acknowledge the role of a healthy lymphatic system in normal wound healing and the adverse outcomes associated with a failing lymphatic system is a serious one which has significant long term consequences if not recognised. Lymphoedema impacts considerably on a patient's quality of life, mobility, and emotional wellbeing, and also leaves the injured limb with an increased risk of recurrent infection and delayed healing (62). It is clinically recognised that patients raise the issue about chronic oedema and diminished sensibility in the leg or foot but a treatment or procedure protocol to remediate this remains absent (58, 63). Studies of short and long term outcomes of lower limb trauma patients show poor functional outcomes and this is of significant concern (64). Correlation of poorer outcomes with lymphatic dysfunction may help to identify patients that are at risk of wound healing complications and implement appropriate management.

1.17 Lymphatic imaging

Ongoing debate regarding the best non-invasive method for observing lymph vessels could be the reason the lymphatic system is often neglected and thus not adequately managed. Considerable advances in lymphatic imaging have been made over the last decade. A useful imaging technique for differential diagnosis is Magnetic Resonance Imaging (MRI). The use of Computed Tomography (CT) and ultrasound imaging techniques can detect structural abnormalities, an indication of a failing lymphatic system (24). It would be highly useful to be able to image functional status of the lymphatic system itself. There are various techniques for determining functional signs including intradermal injection of Patent Blue, radionuclide lymphoscintigraphy with 99mTc labelled human serum albumin or 99mTc sulphur colloid. These are classified as invasive measurements (24). This might contribute to a reticence in their use, as not only is the patient exposed to ionizing radiation, but also the contrast fluid has been found to be associated with nephrogenic systemic fibrosis (57). In view of these negatives, the recent interest in using Indocyanine Green (ICG) seems a sensible and minimal risk alternative and it is these aspects which have led to a recent increase in its popularity in research and clinical practice.

ICG is a water-soluble tricarbocyanine dye which has clinically been used for the imaging of cardiac output, hepatic function, cancer tumour excisions and retinal vascularisation (66). This fluorescing contrast agent binds to proteins and is made visible with excitation light and recorded with a near infrared camera system. ICG is considered a relatively safe and non-invasive technique that provides accurate functional lymphatic imaging. Skin is relatively transparent to ICG with near infrared imaging and a clear real life image can be obtained. The fluorescence of ICG is within the near infrared spectrum range (approximately 800nm) and this makes the dermal lymphatics visible. This research showed repeatability of the test, easy interpretation and it seems to be more cost-effective than lymphoscintigraphy (65). The

usefulness of ICG has been proven in skin and breast cancer sentinel lymph node biopsy as a real-time fluorescence tracer (66-69). Furukawa et al. (2012) used ICG fluorescence lymphography to assist the excision of in-transit metastatic melanoma (65). Holm et al. observed that certain haemodynamic and intrinsic metabolic pathways are invaluable for wound healing and post- operative outcomes and argues the need for clinical studies with fluorescence dye intra-operatively to ensure the best possible outcome for tissue survival and skin viability (70).

1.18 Chapter conclusions

Oedema associated with soft tissue trauma of the lower limbs and its remediation is a normal physiological response. This oedema is linked to lymphatic function but the involvement of the lymphatic system has only been raised sporadically in research studies relating to soft tissue injury. Further, as wound healing progresses there is unavoidable scar tissue formation which can further restrict lymphatic flow, impair function, and impact on its regenerative abilities but this is rarely addressed. The imaging technique, ICG lymphography, may provide information of the superficial lymphatic system pattern in and around reconstructed area. This may improve our understanding of lymphatics response to trauma and may help identify those at risk for the development of lymphoedema.

References

1. Farrow N, Gruen R, Baker A-M, Friend M, Clark P, Hicks A. Response to the Australian Commission for Safety and Quality in Health Care on the 'Australian Safety and Quality Goals for Health Care – Consultation Paper'. Monash University, The Alfred Hospital; 2012.

2. Australian Bureau of Statistics. Resident Population Canberra: Australian Bureau of Statistics; 2015 [Available from: www.abs.gov.au.

3. Kent A, Pearce A. Review of morbidity and mortality associated with falls from heights among patients presenting to a major trauma centre. Journal of Emergency Medicine Australasia. 2006;18(1):23-30.

4. Curtis K, Caldwell E, Delprado A, Munroe B. Traumatic injury in Australia and New Zealand. Australasian Emergency Nursing Journal. 2012;15(1):45-54.

5. Kendrick D, O'Brien C, Christie N, Coupland C, Quinn C, Avis M, et al. The impact of injuries study. Multicentre study assessing physical, psychological, social and occupational functioning post injury - a protocol. BioMedCentral Public Health. 2011;11(963).

6. Bradley C, Harrison JE. Descriptive epidemiology of traumatic fractures in Australia. Canberra: Australian Institute of Health and Welfare; 2004.

7. Millar IL, McGinnes RA, Williamson O, Lind F, Jansson KA, Hajek M, et al. Hyperbaric Oxygen in Lower Limb Trauma (HOLLT); protocol for a randomised controlled trial. BMJ Open. 2015;5(6):e008381.

8. Tovell A, McKenna K, Bradley C, Pointer S. Hospital separations due to injury and poisoning 2009–10. Canberra: Australian Institute of Health and Welfare, 2012 Contract No.: 145.

9. Health A. Caring for the Severely Injured in Australia: Inaugural Report of the Australian Trauma Registry 2010 to 2012. Melbourne: Alfred Health, 2014.

10. Kreisfeld R, Harrison JE. Hospital separations due to injury and poisoning 2005–06. Canberra: Australian Institute of Health and Welfare, 2010 Contract No.: 131.

11. Gotschall CS. The Functional Capacity Index, second revision: morbidity in the first year post injury. International Journal Injury Control and Safety Promotion. 2005;12(4):254-6.

12. O'Toole RV, Castillo RC, Pollak AN, MacKenzie EJ, Bosse MJ. Determinants of patient satisfaction after severe lower-extremity injuries. The Journal of Bone and Joint Surgery. 2008;90(6):1206-11.

13. Sears ED, Burke JF, Davis MM, Chung KC. The influence of procedure delay on resource use: a national study of patients with open tibial fracture. Plastic and reconstructive surgery. 2013;131(3):553-63.

14. Parrett BM, Pribaz JJ. Lower extremity reconstruction. Revista Medica Area Academica De Clinica Las Condes. 2010;21(1):66-75.

15. Scallan JP, H. HV. In vivo determination of collecting lymphatic vessel permeability to albumin: a role for lymphatics in exchange. The Journal of physiology. 2010;588(1):243-54.

16. Michelini S, Amore M, Tapia L, Pattarone GR, Mercado D, Torrisi JS, et al. Embryology. In: Neligan P C, Masai J, Piller NB, editors. Lymphedema Complete medical and surgical management. 1. USA: Taylor and Francis Group; 2016. p. 53-61.

17. Levick JR. Revision of the Starling principle: new views of tissue fluid balance. The Journal of physiology. 2004;557.

18. Koltowska K, Betterman KL, Harvey NL, Hogan BM. Getting out and about: the emergence and morphogenesis of the vertebrate lymphatic vasculature. Development. 2013;140(9):1857-70.

19. Norrmen C, Tammela T, Petrova TV, Alitalo K. Biological basis of therapeutic lymphangiogenesis. Circulation. 2011;123(12):1335-51.

20. Hancock DG. Immune regulation by the peripheral lymphatics. In: Neligan P, Masai J, B. PN, editors. Lymphedema Complete medical and surgical management. 1. USA: Taylor & Francis Group; 2015. p. 163-77.

21. Földi M, Földi E. Földi's textbook of lymphology : for physicians and lymphedema therapists. Strössenreuther RHK, Kubik S, editors. München: Elsevier Urban & Fischer; 2012.

22. Zuther JE. Lymphedema management : the comprehensive guide for practioners. New York: Thieme; 2005.

23. Saladin KS. Anatomy & Physiology :The unity of form and function. New York, N.Y.: McGraw-Hill; 2007.

24. Lee BB, Bergan JJ, Rockson SG. Lymphedema a concise compendium of theory and practice. London: Springer; 2011.

25. Partsch H, Moffatt C. An overview of the science behind compression bandaging for lymphoedema and chronic oedema. Best Practise for the management of lymphoedema Compression Therapy: A position document on compression bandaging [Internet]. 2012 30/03/2013; 2:[12-23 pp.]. Available from: http://www.lympho.org/resources.php.

26. Andrade M. Relationship between fat tissue and lymphangiogenesis. In: Neligan P, Masai J, Piller NB, editors. Lymphedema complete medical and surgical management. 1. USA: Taylor & Francis Group; 2015. p. 135-43.

27. Sajid S, Kazmi H, Stranden E, Kroese AJ, Slagsvold C-E, Diep LM, et al. Edema in the Lower Limb of Patients Operated on for Proximal Femoral Fractures. The Journal of Trauma, Injury, Infection, and Critical Care. 2007;62:701-7.

28. Parrett BM, Matros E, Pribaz JJ, Orgill DP. Lower extremity trauma: trends in the management of soft-tissue reconstruction of open tibia-fibula fractures. Jounal of Plastic and Reconstructive Surgery. 2006;117(4):1315-22.

29. Meiklejohn JA, Heesch KC, Janda M, Hayes SC. How people construct their experience of living with secondary lymphoedema in the context of their everyday lives in Australia. Supportive care in cancer. 2013;21(2):459-66.

30. Macdonald JM, Sims N, Mayrovitz HN. Lymphedema, lipedema, and the open wound: the role of compression therapy. Surg Clin North Am. 2003;83(3):639-58.

31. Armer JM, Hulett JM, Cormier JN, Stewart BR, Wanchai A, Cromwell KD. Lymphedema and its impact on quality of life. In: Neligan PC, Masai J, Piller NB, editors. Lymphedema Complete medical and surgical management. 1. USA: Taylor and Francis Group; 2016. p. 24-41.

32. NBOCC. Review of research evidence on secondary lymphoedema: incidence, prevention, risk factors and treatment. NSW National Breast and Ovarian Cancer Centre., 2008.

33. ANPHA. Obesity: Prevalence trends in Australia. Promoting a healthy Australia. In: health P, editor. Canberra: Australian National Preventive Health Agency; 2014.

34. World Health Organization. International classification of functioning, disability, and health : ICF short version. Geneva: World Health Organization; 2001.

35. Viehoff PB, Potijk F, Damstra RJ, Heerkens YF, van Ravensberg CD, van Berkel DM, et al. Identification of relevant ICF (International Classification of Functioning, Disability and Health) categories in lymphedema patients: A cross-sectional study. Acta Oncologica. 2015;54(8):1218-24.

36. Simon RR, Koenigsknecht SJ, Sharieff GQ. Leg. In: Simon R R, J. KS, editors. Emergency orthopedics. 6th edition ed. New York: McGraw-Hill; 2001. p. 483-7.

37. Faschingbauer M, Meiners J, Schulz AP, Rudolf K-D, Kienast B. Operative Treatment and Soft Tissue Management of Open Distal Tibial Fractures –Pitfalls and Results. European Journal of Trauma and Emergency Surgery Focus on Distal Tibial Fractures. 2009;35:527-31.

38. Gustilo RB, Mendoza RM, Williams DN. Problems in the management of type III (severe) open fractures: a new classification of type III open fractures. The Journal of trauma. 1984;24(8):742-6.

39. Bell A, Templeman D, Wienlein JC. Nonunion of the Femur and Tibia: An Update. The Orthopedic clinics of North America. 2016;47(2):365-75.

40. Zalavras CG, Marcus RE, Levin S, Patzakis MJ. Selected Instructional Course Lectures. Management of Open Fractures and Subsequent Complications. The Journal of Bone and Joint Surgery. 2007;89A(4):883-95.

41. Park J, Yang KH. Treatment of an open distal tibia fracture with segmental bone loss in combination with a closed proximal tibia fracture: a case report. Archives Orthopaedic and Trauma Surgery. 2012;132(8):1121-4.

42. Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. Journal of Internalional Medical Research. 2009;37(5):1528-42.

43. Flour M. Dermatological issues in lymphoedema and chronic oedema. International Lymphoedema Framework Position Document, 2012.

44. Breier G. Lymphangiogenesis in Regenerating Tissue : Is VEGF-C Sufficient? Journal of the American Heart Association: Circulation research. 2005;96:1132-4.

45. Nogami M, Hoshi T, Arai T, Toukairin Y, Takama M, Takahashi I. Morphology of lymphatic regeneration in rat incision wound healing in comparison with vascular regeneration. Leg Med (Tokyo). 2009;11(5):213-8.

46. Wagels M, Rowe D, Senewiratne S, Theile DR. History of lower limb reconstruction after trauma. Australian New Zealand Journal of Surgery. 2013;83(5):348-53.

47. Bui DT, Cordeiro PG, Hu QY, Disa JJ, Pusic A, Mehrara BJ. Free flap reexploration: indications, treatment, and outcomes in 1193 free flaps. Journal of Plastic and Reconstructive Surgery. 2007;119(7):2092-100.

48. Gaboriau HP, Murakami CS. Skin anatomy and flap physiology. Otolaryngologic clinics of North America. 2001;34(3):555-69.

49. Salgado CJ, Moran SL, Mardin S. Flap monitoring and patient management. Journal of Plastic and Reconstructive Surgery. 2009;124(6):295-302.

50. Machens HG, Mailänder P, Pasel J, Lutz BS, Funke M, Siemers F, et al. Flap perfusion after free musculocutaneous tissue transfer: the impact of postoperative complications. Plastic and reconstructive surgery. 2000;105(7):2395-9.

51. Salmi A, Tukiainen E, Härmä M, Asko-Seljavaara S. A prospective study of changes in muscle dimensions following free-muscle transfer measured by ultrasound and CT scanning. Journal of Plastic and Reconstructive Surgery. 1996;97(7):1443-50.

52. Willey M, Karam M. Impact of Infection on Fracture Fixation. Orthopedic Clinics of North America. 2016;47(2):357-64.

53. Starnes-Roubaud MJ, Peric M, Chowdry F, Nguyen JT, Schooler W, Sherman R, et al. Microsurgical Lower Extremity Reconstruction in the Subacute Period: A Safe Alternative. Journal of Plastic and Reconstructive Surgery. 2015;3(7).

54. Slavin SA, Upton J, Kaplan WD, Van den Abbeele AD. An investigation of lymphatic function following free-tissue transfer. Plast Reconstr Surg. 1997;99(3):730-41; discussion 42-3.

55. Khazanchi R, Rakshit K, Bal C, Guleria S, Sinha S, Srivastava A. Evaluation of lymphatic drainage in free flaps by lymphoscintigraphy: a preliminary study. Br J Plast Surg. 1996;49(2):123-8.

56. Szczesny G, Olszewski WL, Gewartowska M, Zaleska M, Gorecki A. The healing of tibial fracture and response of the local lymphatic system. Journal of Trauma. 2007;63(4):849-54.

57. Lohrmann C, Pache G, Felmerer G, Földi E, Schaefer O, Langer M. Posttraumatic edema of the lower extremities: evaluation of the lymphatic vessels with magnetic resonance lymphangiography. Journal of vascular surgery. 2009;49(2):417-23.

58. Cohen MD. Complete Decongestive Physical Therapy in a Patient with Secondary Lymphoedema Due to Orthopedic Trauma and Surgery of the Lower Extremity American Physical Therapy Association 2011;91(11):1618-26.

59. Szczesny G, Olszewski WL. The pathomechanism of posttraumatic edema of the lower limbs: I-- The effect of extravasated blood, bone marrow cells, and bacterial colonizationon tissues, lymphatics, and lymph nodes. Journal of Trauma. 2002;52(2):315-22

60. Szczesny G, Olszewski WL. The pathomechanism of posttraumatic edema of the lower limbs: II--Changes in the lymphatic system. Journal of Trauma. 2003;55(2):350-4

61. Kauhanen MS, Lorenzetti F, Leivo IV, Tukiainen E, Asko-Seljavaara SL. Long-term morphometric and immunohistochemical findings in human free microvascular muscle flaps. Microsurgery. 2004;24(1):30-8.

62. Moffatt CJ, Franks PJ, Doherty DC, Williams AF, Badger C, Jeffs E, et al. Lymphoedema: an underestimated health problem. Journal of the Association of Physicians. 2003;96(10):731-8.

63. Hoogendoorn JM, van der Werken C. Grade III open tibial fractures: functional outcome and quality of life in amputees versus patients with successful reconstruction. Journal of Injury. 2001;32(4):329-34.

64. MacKenzie EJ, Bosse MJ, Pollak AN, Webb LX, Swiontkowski MF, Kellam JF, et al. Long-term persistence of disability following severe lower-limb trauma. Results of a seven-year follow-up. The Journal of Bone and Joint Surgery. 2005;87(8):1801-9.

65. Furukawa H, Hayashi T, Oyama A, Takasawa A, Yamamoto Y. Tailored excision of in-transit metastatic melanoma based on indocyanine green fluorescence lymphography. European Journal of Plastic Surgery. 2012;35(4):329-32.

66. Fujiwara M, Mizukami T, Suzuki A, Fukamizu H. Sentinel lymph node detection in skin cancer patients using real-time fluorescence navigation with indocyanine green: preliminary experience. Journal of Plastic, Reconstructive & Aesthetic Surgery. 2009;62(10):e373-e8.

67. Suami H, Chang D, Skoracki R, Yamada K, Kimata Y. Using indocyanine green fluorescent lymphography to demonstrate lymphatic architecture. International Journal of Lymphoedema. 2012;7(2):25-9.

68. Akita S, Mitsukawa N, Rikihisa N, Kubota Y, Omori N, Mitsuhashi A, et al. Early diagnosis and risk factors for lymphedema following lymph node dissection for gynecologic cancer. Plast Reconstr Surg. 2013;131(2):283-90.

69. Miyashiro I, Hiratsuka M, Kishi K, Takachi K, Yano M, Takenaka A, et al. Intraoperative diagnosis using sentinel node biopsy with indocyanine green dye in gastric cancer surgery: an institutional trial by experienced surgeons. Annals of Surgical Oncology. 2013;20(2):542-6.

70. Holm C, Mayr M, Höfter E, Becker A, Pfeiffer UJ, Mühlbauer W. Intraoperative evaluation of skin-flap viability using laser-induced fluorescence of indocyanine green. British journal of plastic surgeryl. 2002;55(8):635-44.

CHAPTER 2
Imaging the lymphatic system

One of the promising techniques for studying the lymphatic system described in *Chapter 1: Introduction and literature review*, involves imaging with the contrast agent Indocyanine Green (ICG) and a Near-Infrared imaging system. These systems for stimulating fluorescence and recording have been produced by specialist manufacturers. Unfortunately, the costs limit their wider use in research. This *Chapter 2: Imaging the lymphatic system*, describes the development of a ICG lymphatic imaging system. The availability of this, and similar instruments, can make investigations into lymph flow and functioning more feasible in a wider range of laboratories and clinics.

2.1 Background lymphatic imaging

Imaging a delicate biological system that consists of translucent vessels, of small diameter between 2 μm and 10 μm, and that transports mostly clear to milky white fluid is understandably quite challenging. However, imaging of this system is important as we explore its anatomy to better understand its pathology (3).

The first description of the lymphatic system seems to be from approximately 300 BC where reports from Herophilos and later from Erasistratus describe a draining system parallel to the blood vessels (4). It remains arguable whether they truly were describing the lymphatic system or merely observing additional branches of veins, but more descriptions followed, such as the discovery of "fibres between the blood vessels and the nerves" and "white blood" (4). The first reported injections of lymphatic vessels for the purpose of visualisation were performed using wax and suet in the mid-17th century, leading to the discovery and detailed description of lymphatic valves. This was soon followed by the injection of mercury for further exploration of vessel and valve morphology (4). The first full illustration of the lymphatic

vessels in humans was completed in 1653 by Vesling (5). From that time on, the exploration of the structure and function of the lymphatic system proceeded slowly, and we continue to have many unanswered questions regarding this system.

2.2 *Lymphatic imaging techniques*

Over the last century, exploration has continued using a variety of imaging equipment and techniques to determine their capability of imaging lymphatic vessels and nodes, and their ability to provide information about lymphatic structure and function. In relation to lymphatic pathology, imaging techniques are also being applied that can take into account related soft tissue changes. Many of these techniques use contrast agents. Most important for lymphatic vessel imaging are: the particle size of the contrast agent, chemical composition ratio of the contrast agent which means the agents osmolality or concentration of solution, toxicity and number of iodine atoms per molecule, interstitial pressure and anatomical site of the injection (6). Each of these lymphatic imaging techniques, with its applications and limitations, are described below.

2.2.1 *Structures of soft tissue and tissue fluid*

Lymphatic pathology can change soft tissue structure, with these changes being clinically visible and/or palpable (e.g. in lymphoedema), but also possibly being seen as evident by using imaging equipment and techniques.

- *Ultrasound*

Ultrasound is an imaging technique that acquires images through a high or low frequency sound wave transducer and reflected echographic waves (7). Low frequency sound waves are used to image internal organs, whereas, for superficial imaging of the dermis and epidermis, high frequency sound waves are used. Ultrasound is useful in exploring fluid-filled spaces in the case of lymphatic failure, and in exploring soft tissue structure changes, such as increased

adipose tissue in a more advanced stage of lymphoedema. In addition, ultrasound is used to inspect obstructions or enlargements of lymph nodes. Ultrasound is easily available, and non-invasive but requires an experienced operator to prevent misdiagnosis (8).

- *Computed tomography (CT) and single photon emission tomography (SPECT)*

A computed tomographic (CT) scan is similar to ultrasound with both having the ability to image structures; however, a CT scan can penetrate deeper tissues and can also simultaneously image a whole segment. Computed tomography (CT) has been explored, but does not hold a diagnostic significance, in lymphoedema (9).

- *Dual emission X-ray absorptiometry (DEXA or DXA)*

Dual emission X-ray absorptiometry (DEXA or DXA) assesses body composition and soft tissue structure, with a particular focus on adipose tissue. This scan has proven effective in visualising changes in adipose tissue in later stages of lymphoedema (10).

2.2.2 Lymphatic vessel imaging with contrast fluid

The following paragraphs describe how contrast imaging techniques provide accurate information on lymphatic vessel and valve morphology, and lymphatic system functioning.

- *Blue dye*

Sentinel lymph nodes (SLNs) and cutaneous lymph vessels can be visualised with an intradermal or subdermal injection of a blue dye, such as Evans blue, Patent blue (Isosulfan blue), methylene blue or a combination (11). Inspection can be visual using the naked eye or with microscopy.

- *Micro-lymphangiography and X-ray*

To assess dermal lymph capillaries, a fluorescein-labelled human albumin or fluorescein isothiocynate-dextran is injected intradermally. This technique allows only a small field of view (approximately 1 $cm^{2)}$ and is limited to the lymphatic capillaries (12).

- *Indirect lymphography and X-ray*

This technique involves intradermal injection of a radiopaque oily contrast agent in the skin, with this then detected with multiple X-ray images. It is similar to direct lymphography (see below) except there is no need to cannulate the lymphatic vessel (13). This procedure is relatively invasive and is no longer a preferred method for lymphatic imaging in humans.

- *Direct lymphography and X-ray*

After identifying the lymphatic vessels through the skin by using a blue dye injection, a visible lymphatic vessel is directly cannulated and injected with an oily contrast agent. Using an X-ray detection system, an image is formed of the larger lymphatic collectors and lymph nodes. Direct lymphography is invasive; in addition, the oily contrast agent has been reported to create a risk of embolism. This is no longer a preferred lymphatic imaging method in humans (9, 14).

- *Magnetic resonance imaging (MRI)*

Magnetic resonance imaging (MRI) is applied to image lymphatic vessel malformation, tumours related to lymphatic metastasis and enlarged lymph nodes, and to identify the location of fluid areas above the superficial tissue. It has a better soft tissue contrast compared to CT or ultrasound. A low molecular weight Gadolinium/Gadoteridol or iron-based contrast agent is administered intravenously. The small size of the particles allow for cross capillary transport and localise into lymph nodes (15,17).

- *Magnetic resonance lymphangiography (MRL)*

Magnetic resonance lymphangiography (MRL) operates in the same way as MRI but can be used for three-dimensional lymphatic imaging and for sequentially imaging at high spatial resolution. This scan also uses a Gadoteridol-based contrast agent which is administered intracutaneously in the digital webspace of the foot or hand (16, 17).

- *Lymphoscintigraphy*

Lymphoscintography is the most-frequently reported diagnostic tool for exploring lymphatic functioning of the larger lymphatic collectors and lymph nodes, including sentinel lymph nodes (SLNs) (9). The patient is injected in the webspace between the toes or fingers with a radioactive technetium bound to sulphur colloid or albumin. This contrast agent has been developed exclusively for lymphatic imaging. These radio-labelled particles of proteins are detected by a gamma camera which, by tracking these particles in the lymphatics, can give an indication of vessel morphology (18) .

- *Near-infrared (NIR) lymphatic imaging with indocyanine green (ICG)*

Indocyanine green (ICG) was originally proposed as a real-time lymphatic imaging dye for the detection of SLNs in breast cancer-related lymph node biopsy (19). Given its ease of reconstitution, that its administration requires only a small quantity applied intradermally, and its low toxicity, further exploration of ICG has been rapidly undertaken regarding its use for real-time superficial lymphatic vessel imaging through the skin. Possible uses have included investigation of lymphatic anatomy and lymphatic pathologies such as lymphoedema. ICG imaging in human subjects with lymphoedema was first described by Unno et al. in 2007 (20). It was also investigated for the measurement of lymph velocities in porcine models by tracking the ICG bolus travelling upwards within the lymphatic vessel (21). Research in human subjects continued when ICG transit time between the point of injection,

the knee crease and groin was proposed as an assessment of lymphatic vessel functioning (22). This research group compared ICG with the 'gold standard' of lymphatic imaging, the dynamic lymphoscintigram, and concluded that ICG had potential as a novel imaging technique for lymphatic imaging.

2.3 History of indocyanine green (ICG) and near-infrared (NIR) imaging

Indocyanine green (ICG) is a water-soluble tri-carbonate dye with a molecular weight of 775. This dye was first discovered by the Kodak Research Laboratories (Eastman Kodak, Rochester, NY, USA) which utilised ICG as a pigment for NIR photography in 1955 (23). ICG, amongst other dyes, was introduced to Dr Irwin Fox, a cardiologist at the Mayo Clinic, USA, by an Eastman Kodak company official for use in scientific experiments (23). Dr Fox applied ICG in *in vitro* and *in vivo* physiological experiments related to cardiology. In 1956, ICG was officially utilised for medical imaging and it was approved by the Food and Drug Administration (FDA) in the USA in that same year (24). Specifically, ICG was approved as useful for imaging when administered intravenously to determine cardiac output, as initially described by Dr Fox (25). This was followed by applications to determine liver functioning (26), and for ophthalmic angiography (27, 28), as well as having several applications in general intra-operative angiography, such as neurosurgery, trauma surgery and laparoscopic surgery (29). Although Evans blue had been used as a plasma marker since the 1930s, in 1968, ICG was proposed for measuring plasma volume. The measurement of plasma volume is based on vascular administration of an albumin-binding tracer and monitoring its distribution over time (30).

Since ICG has proven useful for lymphatic imaging, diagnostic proposals have arisen regarding its use in the stages of lymphoedema. Yamamoto et al. proposed pattern recognition that could be associated with specific stages of lymphoedema in the extremities (31). They proposed that dilated vessels could be recognised as a '*splash pattern'* (Figure 2-1

a); dermal backflow as a so-called '*stardust pattern' (*Figure 2-1 b); and complete impaired lymph vessels as a '*diffuse pattern*' (Figure 2-1 c). To the end of 2015, there have been over 200 publications regarding ICG application in lymphography, thus attesting to its wide-ranging use as an imaging modality.

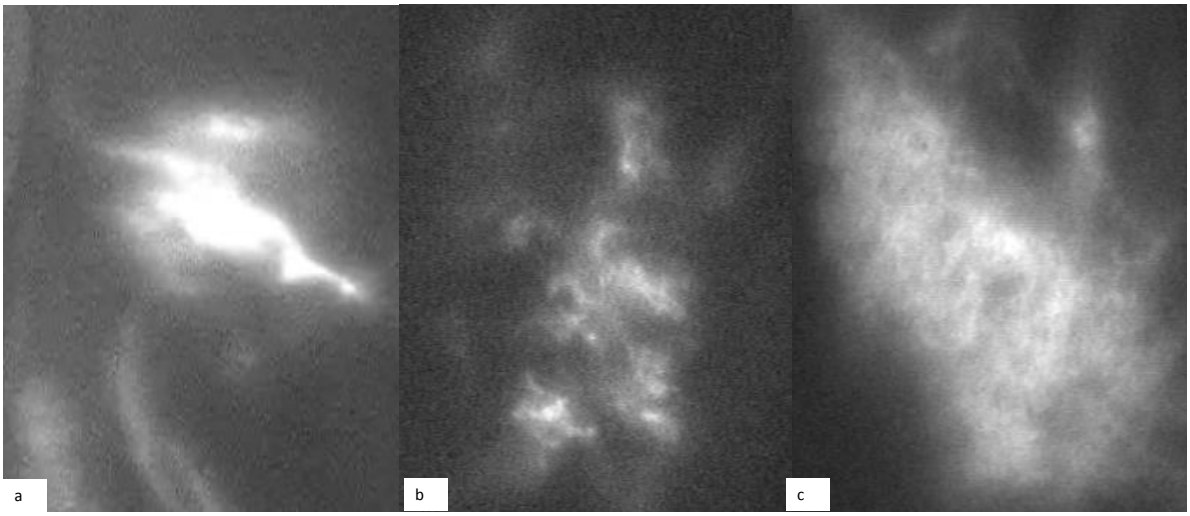

Figure 2-1: Indocyanine green (ICG) pattern in lymphatic impairment based on Yamamoto et al. (28): ICG lymph splash pattern (a); ICG lymph stardust pattern (b); ICG lymph diffuse pattern (c).

2.3.1 Indocyanine green (ICG) specifications

Indocyanine green (ICG) is an unstable macro-molecular dye with a plasma half-life of approximately 120–250 seconds (30, 32). Owing to its small size (<10 nm), it exhibits fast migration and high diffusion properties (33). ICG has sensitivity to photo degradation meaning that exposure to environmental factors, especially white and visible light, influence its deterioration. ICG in aqueous solution should be stocked in complete or near darkness to lessen its deterioration. It binds to proteins and forms aggregates of dye molecules in solution, both of which can affect fluorescence (34).

The intensity and stability of ICG fluorescence change dramatically from weak fluorescence on its own to stable fluorescence when it binds to proteins within blood plasma or lymph (34,

35). Research groups have examined strategies to stabilise ICG as it is unstable and aggregates in solution (36, 37). Binding ICG to lipids seems to provide the best stability, enhancing fluorescence and preventing oxidation (6, 35).

The ICG absorption peak, at which the fluorophore reaches its excited stage, is 780 nm in water and 800 nm in an albumin solution (38). The maximum peak of ICG fluorescence emission is at a wavelength of 832 nm, which is just within the NIR range.

2.3.2 Proteins in lymph

The stability and intensity of ICG fluorescence depend also on the concentration of protein (especially albumin) within the biological tissues. After injection, 95–99% of ICG binds immediately (within 1–2 seconds) to albumin, with the remainder binding to globulins, apolipoproteins and alphaI-lipoprotein (39). The concentration of interstitial proteins available for ICG binding depends on the area of the body and the vascular permeability in that area. Lymph consists of albumin, globulins, and lipoproteins which makes it a favourable combination of proteins for binding with ICG. Free ICG is then taken up by the parenchymal cells in the liver and transported completely (99%) into bile by the toxin-binding properties of the glutathione S-transferase enzymes (Figure 2-2). This is reported to be within 0.5–2 hours of the administration of the intravenous injection (dose-dependent). ICG is not metabolised, nor excreted by the kidneys nor reabsorbed through the enteroenteric circulation (39, 40).

2.3.3 Allergic reactions and reported adverse events

Overall, allergic and adverse reactions to ICG are rare and, if apparent, occur after intravenous administration rather than with intradermal injection. ICG consists of monosodium salt. There is a warning that the iodides within ICG could potentially cause an adverse allergic reaction. However, the level of iodides within ICG is negligible and comparable to the level of iodide intake that we encounter in our daily lives (41). Despite this, patients with thyroid

dysfunction are unable to process iodide normally and will be at a higher risk of an adverse reaction to ICG. Patients who suffer from an overactive thyroid or from benign tumours of the thyroid could be at risk of an adverse response (40).

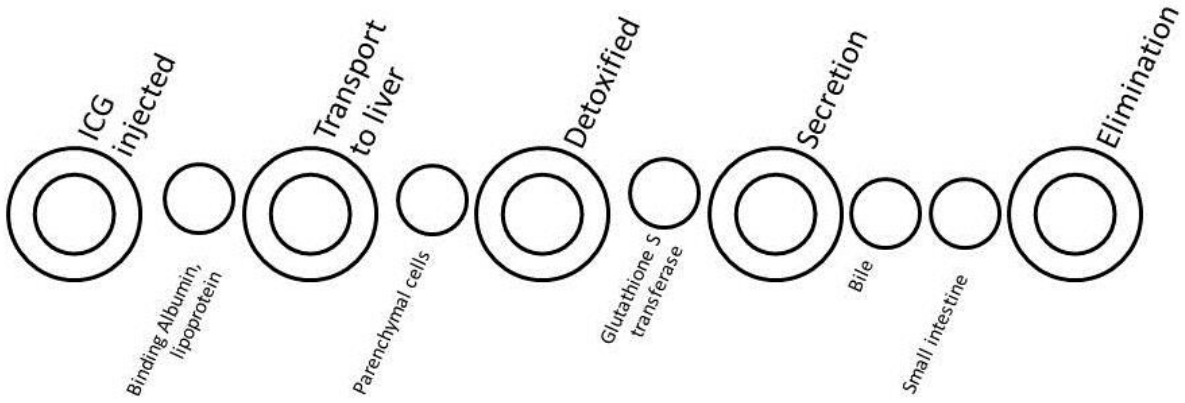

Figure 2-2: Example of ICG elimination process (from left to right).

Sriganesh et al. reported that, in one surgical case of a 42-year-old female operated on for a basilar aneurysm, there was a reduction in the blood oxygen saturation reading from 99% to 96% and another drop to 94% during the procedure. This was based on a intravenous administration of the standard dose of 0.26mg / Kg. Although only one case is described in their study, additional similar cases are mentioned but further details are not provided (42). Gashev et al. reported uncontrollable contractility induced by ICG. They isolated and cannulated mesenteric lymphatic vessels and administered non-irradiated ICG to a small number of these vessels with unknown controls, and warned of altered lymphatic function with ICG usage (43). Due to only this single study mentioning contractility issues in an apparently small number of animals and the nature of the experiment, it is likely that this finding is not translatable to human experiments.

A comprehensive review published by Marshall et al. in 2010 concluded that the incidence of mild adverse reactions was 0.05%, with an incidence of 0.05% for severe adverse reactions

and no deaths reported after 1,923 procedures. In another study reported in that review, the ICG administration for angiography resulted in adverse events in 0.07% of 2,820 patients (44). Further adverse events reported are related to intravenous administration of larger doses of ICG, or relate to patients with existing comorbidities, high-risk surgeries such as open heart surgery or aneurysm-related interventions with ICG (44).

2.3.4 Reconstitution and quantity of indocyanine green (ICG)

The quantity of ICG dye needed to bind to albumin and excite fluorescence is dependent on the nature of the diagnostic test. For tests related to blood flow, the quantity is standardised at 5 mg per kg weight of the patient. For lymphatic imaging, currently there is no consensus: generally, a small quantity, approximately 0.1 mL to 0.2 mL, of manufacturer-recommended solution (25 mg/5 mL–5 mg/1 mL) is administered in the tissue spaces (intradermally or subcutaneously). Marshall et al. reported eight clinical studies that administered between 0.00031 mg and 15 mg of ICG intradermally for lymphatic imaging. This same review reports another 10 clinical studies that administered between 1.5 mg and 25 mg reconstituted ICG subcutaneously for lymphatic imaging (44). Studies on ICG have used a large range of ICG volume and concentration, with no consistent ICG quantity apparently recommended for lymphatic imaging.

The injection dose seems to be dependent on the choice of injection syringe and method of injection, that is, intradermal versus subcutaneous. Intradermal injection allows the syringe to be on a 10° to 15° angle which can cause some back pressure due to the density of the dermal tissues. Therefore, administration of the contrast agent must be slow, based on perceived resistance from the tissue, with the volume limited to approximately 0.1 mL.

Subcutaneous injection of ICG means that it penetrates the epidermis and the dermis, and enters the subdermal or subcutaneous tissue. In this case, the syringe can be angled at 45° and, as the tissue is less dense, it is much easier to inject a contrast agent. A larger quantity

can be injected as there is lower resistance. However, should the subcutaneous injection be too deep, the ICG is likely to be absorbed by the larger lymphatic collectors. Then, if the ICG is visible at all, it will present a blurry and diffuse image of the lymph vessel. This is due to the penetration depth limitations of the NIR imaging system and excitation light. The injection method applied for ICG lymphography is therefore critical, as it affects the quality of the image.

2.3.5 The principle of ICG fluorescence imaging

The wavelength of the emitted fluorescence of ICG in biological tissues is not within visible light. To observe structures with ICG requires an imaging system, an excitation light source and an imaging camera with a specific filter that is suitable for detecting the emitted fluorescence. Both excitation and fluorescence light are within the NIR range where light absorption by haemoglobin (and water) is small; therefore, light can travel quite long distances (Figure 2-3). The NIR range also results in less likely scattering from the skin surface, minimal absorption and good penetration (2).

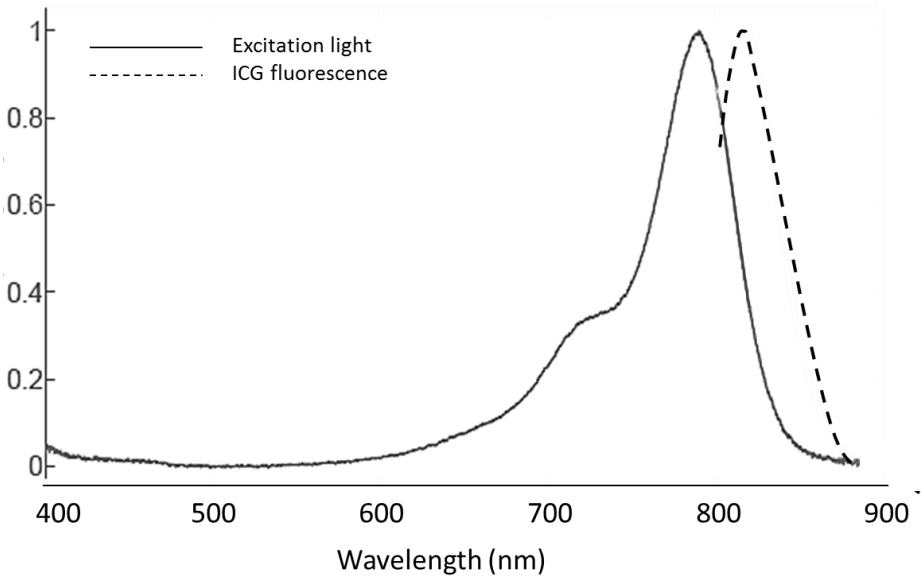

Figure 2-3: Indocyanine green (ICG) wavelength and excitation wavelength (1).

The energy needed for the fluorophore ICG to absorb and emit fluorescence is between wavelengths of 760 nm and 785 nm (Figure 2-4). This depends on the binding properties as binding to plasma proteins can increase the absorption spectrum by 25 nm (34). ICG will reach its excited state and will fluoresce from approximately 805 nm to a maximum peak of 832 nm. To isolate the fluorescence emission of a single fluorophore, such as ICG, a photosensitive detector and a filter are needed to provide real-time imaging of fluorescence. The ideal filter for the single fluorophore isolation is a long-pass filter of the right wavelengths: the ideal detector device is one that can visualise in the NIR range in order to record emitted photons.

ICG fluorescence emission will be activated by an excitation light source, projected on the area of interest, which can be laser or high powered light emitting diodes (LEDs). Using a light source with narrow spectrum, such as laser, there is no need for excitation filter. This is different when using LED light as this is usually a bell shaped spectrum. The tissue absorbs the excitation wavelength and emits fluorescence of the injected substance. When ICG emits fluorescence it can only be detected by a camera within the NIR range through an appropriate filter. This filter will ensure the excitation light scatter or other unwanted wavelengths is blocked from the detection camera. This ensures a maximum fluorescence detection and recording, and minimum excitation leakage (29).

2.3.6 Custom-made imaging camera

Commercially available imaging devices are costly and difficult to source in Australia. As the specifications and requirements for this type of system were clear from the literature, it was proposed that a camera system be developed with the Biomedical Engineering team at Flinders Medical Centre, South Australia.

Previously published details of custom-made cameras related to lymphatic imaging were sought and are summarised in Table 2-1.

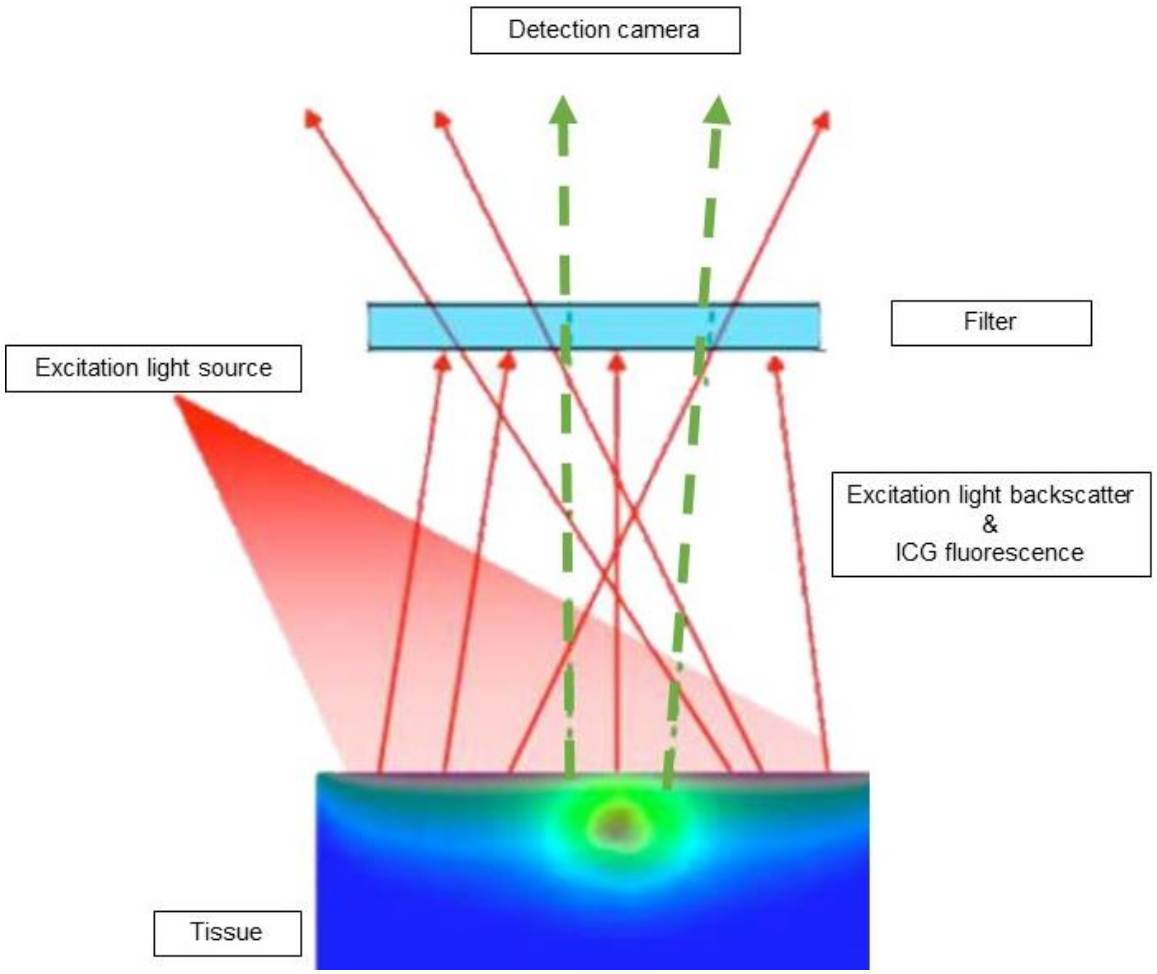

Figure 2-4: ICG fluorescence process. Exiting excitation light source onto the area of interest (tissue), ICG will be activated and emit fluorescence, this will be filtered and detected by the imaging or detection camera. Modified from original image by Jacob et al (2007) (30).

The image system was designed to excite ICG with a fixed bandwidth high power excitation light source (700–830 nm), and to record a real-time video of the resulting fluorescence (760–870 nm; NIR spectrum) as the dye travelled through the lymphatic system.

To select the correct excitation light, in terms of its wavelength and power properties, a comparison was made between a powerful LED and a laser torch originally designed to be used on a hunting rifle. The sourced LED was outside the required wavelength and too weak in intensity for ICG excitation. Therefore, the decision was made to proceed with the laser torch (Yukon Optics, L-808S IR, with a middle peak of 789 nm).

Further testing of the L-808S IR laser torch was conducted to explore any effects from the transmission of laser through different materials (Figure 2-5). No deviation from the peak excitation intensity was visible, and the L-808S IR laser torch was deemed suitable for further exploration.

Table 2-1: Published custom-made imaging devices related to lymphatics. CCD = charge-coupled device; SLN = sentinel lymph node.

Reference	Camera specifications	Excitation light source	Experiment
Kitai et al. **(19)**	CCD, Prototype Hamamatsu	LED	Human, SLN detection
Tanaka et al. **(45)**	NIR CCD, Prototype FLARE	LED	Human, SLN detection
Sevick-Muraca et al. **(21)**	Intensified CCD	Laser	Human, SLN detection and lymphatic mapping
Weiler et al. **(46)**	CCD	Laser	Tissue phantom and animal. Lymphatic vessel
Behm **(2)**	CCD	Laser	Tissue phantom
Hirche et al. **(47)**	CCD, Prototype Fluobeam	Laser	Animal model, lymph nodes detection and lymph vessel imaging

2.3.7 Camera and filter specifications

For the detector in this system, a security video camera was used. The camera contained a charge-coupled device (CCD). The CCD contained an array of photosensitive capacitors and was sensitive from the visible to NIR regions of the electromagnetic spectrum. An aspherical infrared (IR) varifocal lens was used to focus the incoming light onto the detector array, with a long-pass filter used to select the peak emission and avoid flooding of the detector.

A precision long-pass 850 nm 12.5 mm filter (Edmund Optics) was inserted between the CCD camera and the lens.

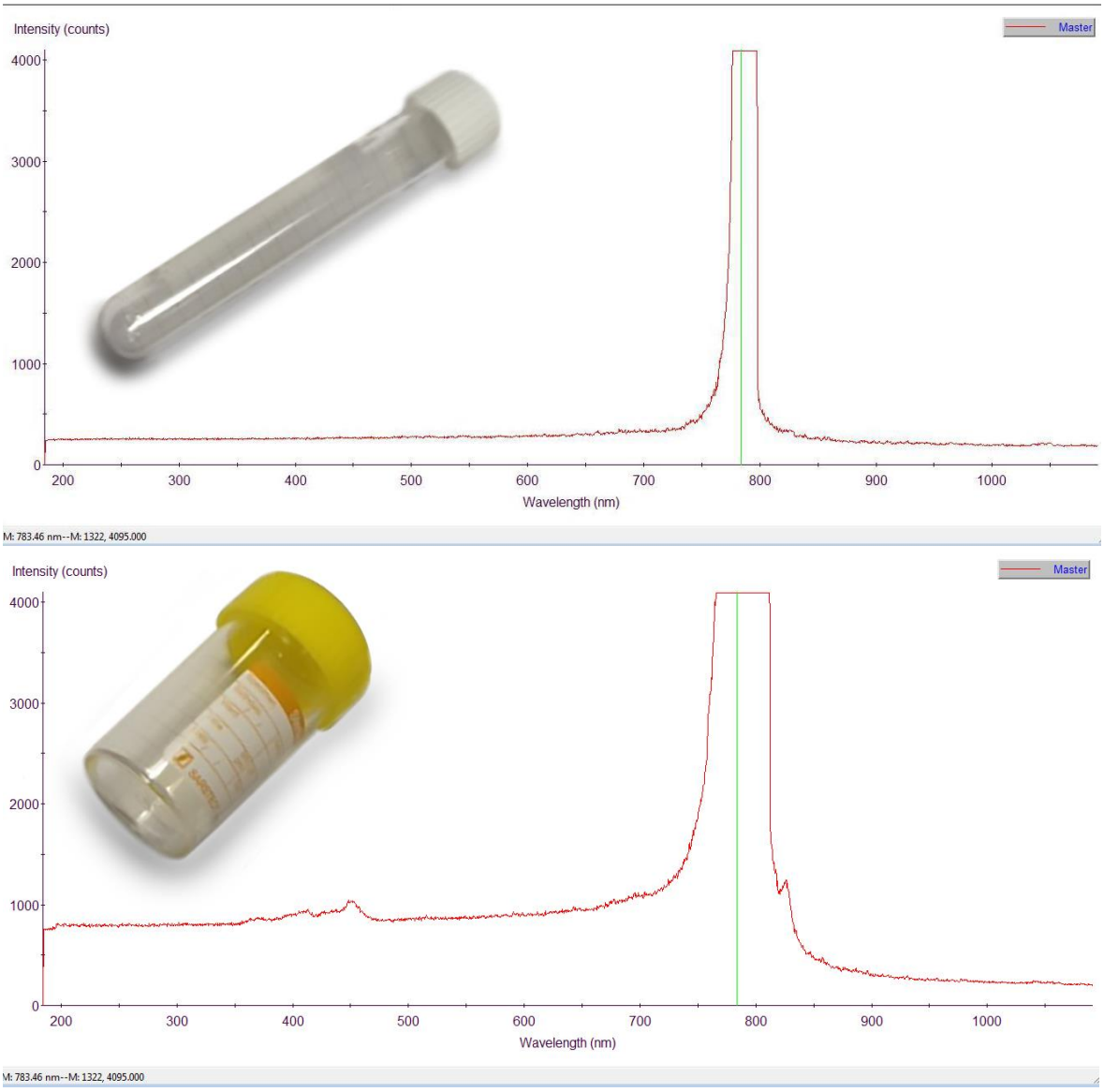

Figure 2-5: Different materials tested for laser penetration. Top glass vial with white lid, bottom plastic vial labelled with yellow lid.

2.3.8 Initial in vitro testing for fluorescence

Initial testing was performed to ensure that the custom-made device was detecting the fluorescence and did not receive interference from the excitation light, thus proving that the filters were working correctly. A few drops of reconstituted ICG were administered to full-

cream milk (chosen for its protein content) so the ICG could bind and emit fluorescence. Then, the milk with fluorescence was placed in several vials and, to mimic a linear lymphatic vessel, in a silicone tube. The tube was covered with one to several layers of 5 mm thickness silicone bandage to mimic penetration through skin and to detect scattering (Figure 2-6 a). The intensity was evident, thus concluding that the correct laser excitation wavelength and strength were being used, and that the filter and camera system were able to detect fluorescence without adverse interference from the excitation light source (Figure 2-6 b). Further fluorescence testing was performed with four one-way rubber sealed vials of 6 mL of expired human plasma samples. Injection through the seal with three drops of reconstituted ICG (PULSION Medical Systems: 1 mg/1 mL) showed immediate fluorescence.

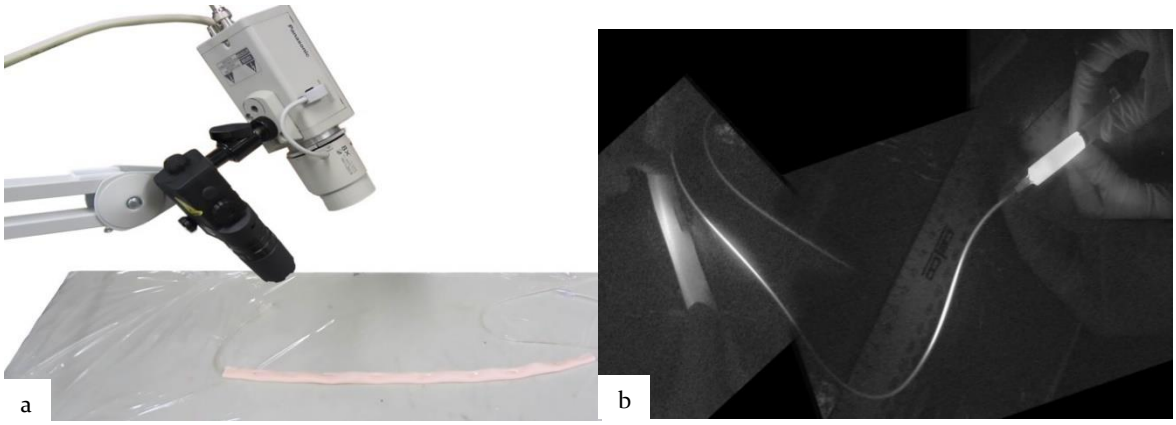

Figure 2-6: Camera set up and tube covered with silicone (a). Injection of ICG activated with milk into the tube (b).

These tests proved the effectiveness of the laser and the capacity of the camera: steps were taken to enhance the fluorescence intensity by adding more laser torches and developing a more comprehensive imaging system.

The complete low cost custom-made pilot imaging system consisted of an image head, positioning frame, trolley and laptop computer with video capture software (Figure 2-7). The total costs of materials are specified in Appendix A

The image head consists of:

- Video camera
- Aspherical IR varifocal lens mounted on the camera
- Long-pass filter with custom mounting
- Four laser diode torches
- Controlled white light source.

Figure 2-7: Close-up of camera imaging head and the whole imaging system set-up

2.3.9 Ex vivo experiment (porcine model)

To test the visibility, intensity and penetration of fluorescence through skin, this study used four porcine hind legs and two ears sourced from the large animal research facility of the South Australian Health and Medical Research Institute (SAHMRI), Gilles Plains, South Australia.

On separate occasions, two similar 12-week-old and 40 kg porcines were injected with 25,000 units of heparin and ketamine four minutes prior to culling. The heparin and ketamine allowed the remaining fluid and tissue to remain relatively soft and stable for approximately four hours.

In the Animal House facility of Flinders University's School of Medicine, the porcine hind legs were injected intradermally between the hooves with reconstituted ICG (PULSION Medical Systems: 1 mg/1 mL), 0.5 mL in volume, with an insulin syringe. During operation of the ICG fluorescence lymphatic vessel imager, all external light sources were switched off to avoid flooding the detector and to avoid interference in the captured images. A controlled white light source was used intermittently, providing gentle illumination in the area of interest prior to and during imaging, to visualise the outline of the porcine leg without flooding the detector and corrupting the resultant image.

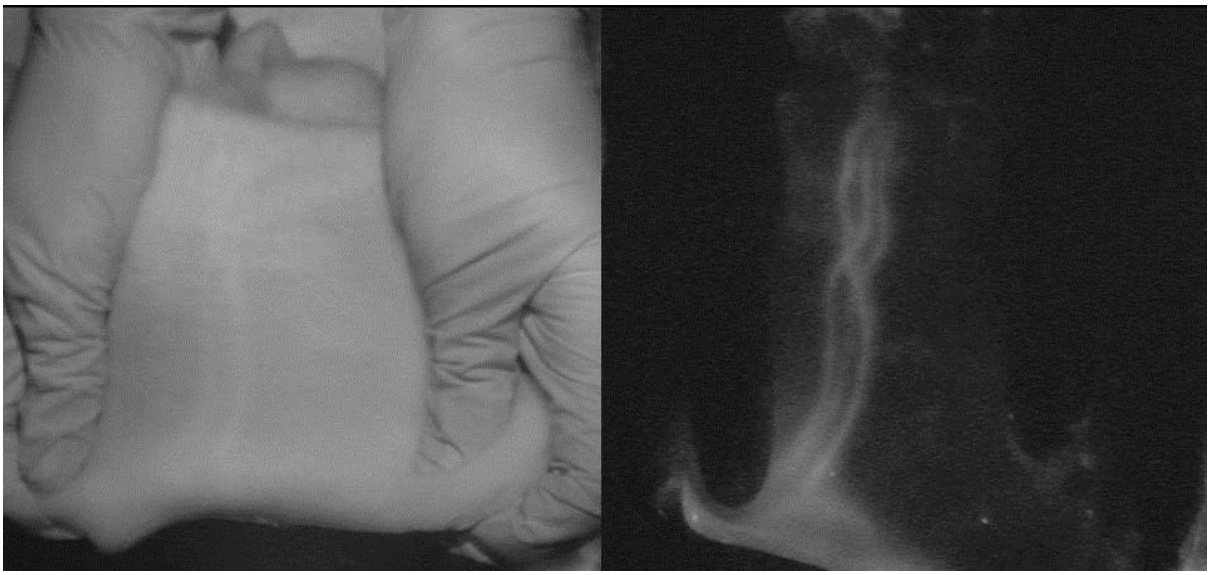

Figure 2-8: Porcine tissue testing with white light and without white light.

Manual manipulations mimicking manual lymphatic drainage, including moving the skin and the joints, were needed to gently push the ICG through the lymphatic vessels. After manipulation, the image system was positioned 300 mm away from the site of interest, the

imager's excitation lasers were energised and images of fluorescing dye were recorded for 20–30 minutes. ICG fluorescence was detected and recorded immediately at the injection site, while fluorescence was detected and recorded in superficial lymph vessels two minutes post-ICG injection. After imaging of the porcine leg, skin and tissue were removed (~0.5cm thickness) of a specific area of interest with clear lymph vessels (Figure 2-8). This was to explore if any more, somewhat deeper, lymphatics were visible after removing tissue. But this was not the case, and resulted in areas of ICG fluorescence caused by contamination, however the lymph vessels in the extracted tissue remained intact.

2.3.10 Further developments of the lymphatic vessel imaging system

The amount of excitation light was increased by attaching four more laser torches to the image head. The image head was mounted on an adjustable positioning frame which allowed it to be moved in three planes during the imaging procedure: distal to proximal (along the length of the leg), laterally (across the leg and between left and right legs), as well as up and down (for focusing). This allowed the image head to be positioned in the start position and for it to move smoothly when tracking the uptake and fluorescence of the dye travelling in the lymphatics of the lower leg. The complete system was mounted on a trolley to ease its transportation to, and positioning within, the research space (Figure 2-9).

Figure 2-9: Image head with eight laser torches and the imaging system set-up.

2.4 Discussion

The ICG fluorescence vessel imager used laser diodes to excite ICG and induce fluorescence as they provide a narrowband light source. While a similar result could be achieved with a wide-band light source and narrowband filter (general approach to fluorescence imaging before LEDs and laser diodes became commonplace), laser diodes represented a simpler and cheaper option with low energy and heat dissipation requirements. Due to the use of narrowband light sources in the design of the ICG imager, a need for background lighting to identify the outline of limbs and location of fluorescence was required. The controlled white light source was used for this purpose.

2.5 Chapter conclusions

The detection of fluorescence from superficial lymph vessels within 2 min of dye injection into porcine legs indicated that laser diode flashlights can generate excitation light with sufficient intensity for fluorescence imaging applications, and a video camera can capture the fluorescence to produce suitable images to map lymphatic pathways.

The development of this custom made system allowed ICG imaging to be accessible. As costs drop, technology improves and ICG imaging is accepted as a diagnostic aid, commercial systems are anticipated to become more accessible to more laboratories and clinics.

References

1. Pallotta O, van Zanten M, McEwen M, Burrow L, Beesley J, N. P. Development and validation of a custom made indocyanine green fluorescence lymphatic vessel imager. Journal of Biomedical Optics. 2015;20(6):066003-.

2. Behm P. Experimental set-up for near infrared fluorescence measurements during surgery. Measurements on ICG phantom using fibre-optical based spectros-copy and camera imaging system. [Bachelor Thesis]: Linköping University, Sweden; 2013.

3. Swartz MA. The physiology of the lymphatic system. Advanced Drug Delivery Reviews. 2001;50:3-20.

4. Loukas M, Bellary SS, Kuklinski M, Ferrauiola J, Yadav A, Shoja MM, et al. The lymphatic system: a historical perspective. Journal of Clinical Anatomy. 2011;24(7):807-16.

5. Chikly B. Who discovered the lymphatic system? Journal of Lymphology. 1997;30:186-93.

6. Jeong H-S, Chang-Moon L, Su-Jin C, Eun-Mi K, Hyosook H, Kyung SN, et al. The effect of mannosylation of liposome-encapsulated indocyanine green on imaging of sentinel lymph node. Journal of liposome research. 2013;23(4):291–7.

7. Tassenoy A, De Mey J, De Ridder F, Van Schuerbeeck P, Vanderhasselt T, Lamote J, et al. Postmastectomy lymphoedema: different patterns of fluid distribution visualised by ultrasound imaging compared with magnetic resonance imaging. Journal of Physiotherapy. 2011;97(3):234-43.

8. Kleinerman R, Whang TB, Bard RL, Marmur ES. Ultrasound in dermatology: Principles and applications. Journal of the American Academy of Dermatology. 2012;67(3):478-87.

9. Földi M, Földi E. Földi's textbook of lymphology : for physicians and lymphedema therapists. Strössenreuther RHK, Kubik S, editors. München: Elsevier Urban & Fischer; 2012.

10. Brorson H, Ohlin K, Olsson G, Karlsson MK. Breast cancer-related chronic arm lymphedema is associated with excess adipose and muscle tissue. Lymphatic research and biology. 2009;7(1):3-10.

11. Tsopelas C, Sutton R. Why certain dyes are useful for localizing the sentinel lymph node. Journal of Nuclear Medicine. 2002;43(10):1377-82.

12. Stanton AW, Kadoo P, Mortimer PS, Levick JR. Quantification of the initial lymphatic network in normal human forearm skin using fluorescence microlymphography and stereological methods. Microvascular research. 1997;54(2):156-63.

13. Partsch H. Assessment of abnormal lymph drainage for the diagnosis of lymphedema by isotopic lymphangiography and by indirect lymphography. Clinics in dermatology. 1995;13(5):445-50.

14. Fuchs WA. Complications in lymphography with oily contrast media. SRAD Acta Radiologica [Old Series]. 1962;57(6):427-32.

15. Zhang F, Niu G, Lu G, Chen X. Preclinical Lymphatic Imaging. Molecular Imaging and Biology. 2011;13(4):599-612.

16. Lohrmann C, Pache G, Felmerer G, Földi E, Schaefer O, Langer M. Posttraumatic edema of the lower extremities: evaluation of the lymphatic vessels with magnetic resonance lymphangiography. Journal of vascular surgery. 2009;49(2):417-23.

17. Liu N, Lu Q, Liu P, Wu X, Wang B. Comparison of radionuclide lymphoscintigraphy and dynamic magnetic resonance lymphangiography for investigating extremity lymphoedema. British Journal of Surgery. 2010;97:359-65.

18. Lee BB, Bergan JJ, Rockson SG. Lymphedema a concise compendium of theory and practice. London: Springer; 2011.

19. Kitai T, Inomoto T, Miwa M, Shikayama T. Fluorescence navigation with indocyanine green for detecting sentinel lymph nodes in breast cancer. International Journal of Breast Cancer. 2005;12(3):211-5.

20. Unno N, Inuzuka K, Suzuki M, Yamamoto N, Sagara D, Nishiyama M, et al. Preliminary experience with a novel fluorescence lymphography using indocyanine green in patients with secondary lymphedema. Journal of vascular surgery. 2007;45(5):1016-21.

21. Sharma R, Wendt JA, Rasmussen JC, Adams KE, Marshall MV, Sevick-Muraca EM. New horizons for imaging lymphatic function. Annals of the New York Academy of Sciences. 2008;1131(01):13-36.

22. Unno N, Nishiyama M, Suzuki M, Yamamoto N, Inuzuka K, Sagara D, et al. Quantitative lymph imaging for assessment of lymph function using indocyanine green fluorescence lymphography. European Journal of Vascular and Endovascular Surgery. 2008;36(2):230-6.

23. Reuben A. Landmarks in hepatology: never say dye. Journal of Hepatology. 2004;39(1):259-64.

24. Fickweiler S, Szeimies RM, Bäumler W, Steinbach P, Karrer S, Goetz AE, et al. Indocyanine green: intracellular uptake and phototherapeutic effects in vitro. Journal of Photochemistry and Photobiology. 1997;38(2-3):178-83.

25. Fox IJ, Brooker LG, Heseltine DW, Essex HE, Wood EH. A tricarbocyanine dye for continuous recording of dilution curves in whole blood independent of variations in blood oxygen saturation. Proceedings of the Staff Meetings of the Mayo Clinic. 1957;32(18):478-84.

26. Cherrick GR, Stein SW, Leevy CM, Davidson CS. Indocyanine green: observations on its physical properties, plasma decay, and hepatic extraction. The Journal of clinical investigation. 1960;39:592-600.

27. Flower RW, Hochheimer BF. Indocyanine green dye fluorescence and infrared absorption choroidal angiography performed simultaneously with fluorescein angiography. Johns Hopkins Medical Journal. 1976;138(2):33-42.

28. Choromokos E, Kogure K, David NJ. Infrared absorption angiography. Journal of the Biological Photographic Association. 1969;37(2):100-4.

29. Alander JT, Kaartinen I, Laakso A, Patila T, Spillmann T, Tuchin VV, et al. A review of indocyanine green fluorescent imaging in surgery. International Journal of Biomedical Imaging. 2012;2012:940585.

30. Jacob M, Conzen P, Finsterer U, Krafft A, Becker BF, Rehm M. Technical and physiological background of plasma volume measurement with indocyanine green: a clarification of misunderstandings. Journal of applied physiology. 2007;102(3):1235-42.

31. Yamamoto T, Narushima M, Doi K, Oshima A, Ogata F, Mihara M, et al. Characteristic indocyanine green lymphography findings in lower extremity lymphedema: the generation of a novel lymphedema severity staging system using dermal backflow patterns. Journal of Plastic and Reconstructive Surgery. 2011;127(5):1979-86.

32. Saxena V, Sadoqi M, Shao J. Degradation kinetics of indocyanine green in aqueous solution. Journal of Pharmaceutical Sciences. 2003;92(10):2090-7.

33. Cousins A, Thompson SK, Wedding AB, Thierry B. Clinical relevance of novel imaging technologies for sentinel lymph node identification and staging. Journal of Biotechnology Advances. 2014;32(2):269-79.

34. Landsman ML, Kwant G, Mook GA, Zijlstra WG. Light-absorbing properties, stability, and spectral stabilization of indocyanine green. Journal of applied physiology. 1976;40(4):575-83.

35. Kraft JC, Ho RJ. Interactions of indocyanine green and lipid in enhancing near-infrared fluorescence properties: the basis for near-infrared imaging in vivo. Journal of Biochemistry. 2014;53(8):1275-83.

36. Proulx ST, Luciani P, Derzsi S, Rinderknecht M, Mumprecht V, Leroux JC, et al. Quantitative imaging of lymphatic function with liposomal indocyanine green. Journal of Cancer Research. 2010;70(18):7053-62.

37. Proulx ST, Luciani P, Christiansen A, Karaman S, Blum KS, Rinderknecht M, et al. Use of a PEG-conjugated bright near-infrared dye for functional imaging of rerouting of tumor lymphatic drainage after sentinel lymph node metastasis. Journal of Biomaterials. 2013;34(21):5128-37.

38. Philip R, Penzkofer A, Baumler W, Szeimies R, Abels C. Absorption and fluorescence spectroscopic investigation of indocyanine green. Journal of Photochemistry and Photobiology. 1996;96(1):137-48.

39. ICG-PULSION. Indocyanine green monosodium salt powder for solution for injection. Munich, Germany: PULSION Medical Systems, 2008.

40. PULSION-Medical-Systems. Summary of product characteristics, specifications sheet. Munich, Germany: PULSION Medical Systems; 2007.

41. Hehrmann R. Indocyanine Green Dye. There is no allergy against indocyanine green dye based on iodine. In: AG PMS, editor. Munich, Germany: Pulsion Medical Systems; 2009.

42. Sriganesh K, Vinay B, Bhadrinarayan V. Indocyanine green dye administration can cause oxygen desaturation. Journal of Clinical Monitoring and Computing. 2013;27(3):371.

43. Gashev AA, Nagai T, Bridenbaugh EA. Indocyanine green and lymphatic imaging: current problems. Journal of Lymphatic Research and Biology. 2010;8(2):127-30.

44. Marshall MV, Rasmussen JC, Tan IC, Aldrich MB, Adams KE, Wang X, et al. Near-Infrared fluorescence imaging in humans with indocyanine green: A review and update. The Open Surgical Oncology Journal. 2010;2:12-25.

45. Tanaka E, Choi HS, Fujii H, Bawendi MG, Frangioni JV. Image-guided oncologic surgery using invisible light: Completed pre-clinical development for sentinel lymph node mapping. Annals of Surgical Oncology. 2006;13(12):1671-81.

46. Weiler M, Kassis T, Dixon JB. Sensitivity analysis of near-infrared functional lymphatic imaging. Journal of Biomedical Optics. 2012;17(6):1-11.

47. Hirche C, Engel H, Kolios L, Cognie J, Hünerbein M, Lehnhardt M, et al. An experimental study to evaluate the Fluobeam 800 imaging system for fluorescence-guided lymphatic imaging and sentinel node biopsy. Journal of Surgical Innovation. 2013;20(5):516-23.

CHAPTER 3
Real-time lymphatic imaging in mice

The development of the custom made ICG imaging system, as described in *Chapter 2: Imaging the lymphatic system,* enables studies into the functional lymphatic system of humans and animals. This *Chapter* will explore lymphatic functional imaging in small animals (*Gata2* heterozygous and wildtype mice). This provided information about the use of ICG imaging in small laboratory animals, including lymph vessel pulsation rates, and flow rates into the popliteal lymph node.

3.1 Background lymphangiogenesis

The origins of and mechanisms regulating lymphatic vessel growth and development, or lymphangiogenesis, have been of interest for many decades. It is important to explore the cellular, molecular and genetic mechanisms underlying lymphangiogenesis in order to understand how the lymphatic vasculature is "built" during development and also to gain insight to how lymphangiogenesis is disrupted in lymphovascular disorders.

Lymphatic vessels sprout from the lymph hearts or sacs. This was first described by Florence Sabin as early as 1902 in pig embryos, and more recently confirmed by Wigle and Oliver in 1999 (1, 2). Sabin showed that lymphatics originate by budding from the cardinal vein, continue to develop as lymph sacs and sprout in the periphery to form the lymphatic plexus (1-4). It is now clear with advances in research that development of the vascular and lymphatic system are separate and independent processes (1, 4). However, if the blood vascular system does not develop, the lymphatic system cannot develop either, so the systems are interdependent (5).

Several transcription factors play key roles in regulating lymphatic development and differentiating lymphatic endothelial cells (LEC) from blood endothelial cells (BEC) (6). One

important transcription factor, GATA binding protein 2 (*Gata2*), has recently been found to be critical in the initiation of lymphatic vessels and particularly lymphatic valve formation where the *Gata2* presence is measured in high levels (7). Initial studies documented *Gata2* mRNA levels to be elevated almost 10 fold in LECs compared with the BECs in endothelial cells isolated from the skin of embryonic mice and found that *Gata2* protein was present at high levels in the endothelial cells that comprise lymphatic vessel valves (7). In mice with selective deletion of *Gata2* in lymphatic endothelium, blood was found within the lymphatic vasculature, suggesting that lymphvenous valve formation was impaired. Lymphatic vessel valve development was also impaired in these mice; a detailed investigation of mutant mice revealed that *Gata2* is required for the initiation of lymphatic vessel valve development and for the maintenance of lymphatic vessel valve architecture and function (7). Emphasising the importance of *Gata2* in the lymphatic vasculature, heterozygous germline mutations in *Gata2* were recently found to underlie a human primary lymphoedema syndrome known as Emberger Syndrome, characterised by lymphoedema and predisposition to myelodysplasia and acute myeloid leukaemia (7, 8) .

3.2 Gata2 heterozygous mice

The discovery of the importance of *Gata2* in the lymphatic vasculature and in particular, in valve development and function has been a large part of recent work by Professor Natasha Harvey and her team at the Lymphatic Development Laboratory, University of South Australia & SA Pathology. In order to define the roles of *Gata2* in lymphangiogenesis, the Lymphatic Development Laboratory developed a *Gata2* mutant mouse model. While deletion of both copies of the *Gata2* gene results in embryonic lethality, heterozygous *Gata2* mice survive to adulthood, similarly to patients with heterozygous *Gata2* mutations. Here, we have utilised heterozygous *Gata2* mice as a model to investigate the consequences of *Gata2* loss of function on lymphatic vascular transport.

3.3 Imaging lymphatic vessel function in Gata2 heterozygous mice

Imaging lymphatic vessel morphology during embryonic development and in adult mice provides invaluable information regarding the structure of the lymphatic vasculature, but does not provide a measure of lymphatic vessel function. Acquiring images of the functioning of lymphatic vessels in *Gata2* mutant mice would dramatically increase the understanding of how structural abnormalities impact on lymphatic vessel function and provide insight to features such as stagnating lymphatic flow, backflow of lymph due to dysfunctioning valves and the presence of subclinical lymphoedema. A comparison of imaging data between *Gata2* heterozygotes and normal wildtype mice will improve our understanding of early stage lymphatic vessel failure and its patterns, which could be translated to human disease.

Lymphatic vessel transport function is currently investigated by imaging the transport of Evans Blue dye through the lymphatic vasculature to the thoracic duct. Approximately 10 microliters of Evans Blue is injected sub dermally into the footpad of both hind paws of anaesthetised mice. The injected mouse is humanely killed with CO^2 15 minutes post Evans Blue injection. The mouse is then carefully dissected to follow the passage of Evans Blue dye from the injection site via lymphatic vessels to local draining lymph nodes (Figure 3-6 a). Extreme care is taken not to damage the fragile lymphatic vessels during dissection to allow an accurate image of the status of the lymphatic vessels and their overall morphology. Studies undertaken by the Lymphatic Development Laboratory in *Gata2* heterozygous mice to date have revealed that the thoracic ducts of these mice appear distended compared to their wild-type counterparts and that in some cases, the transport of Evans Blue dye to the thoracic duct appeared to be delayed (Figure 3-6 b) (7). Though informative, these studies do not provide a quantitative readout of lymphatic vessel function in real time. The goal of this study was to investigate the use of Indocyanine Green (ICG) for less invasive, real time lymphatic imaging of lymphatic vessel transport function in mice.

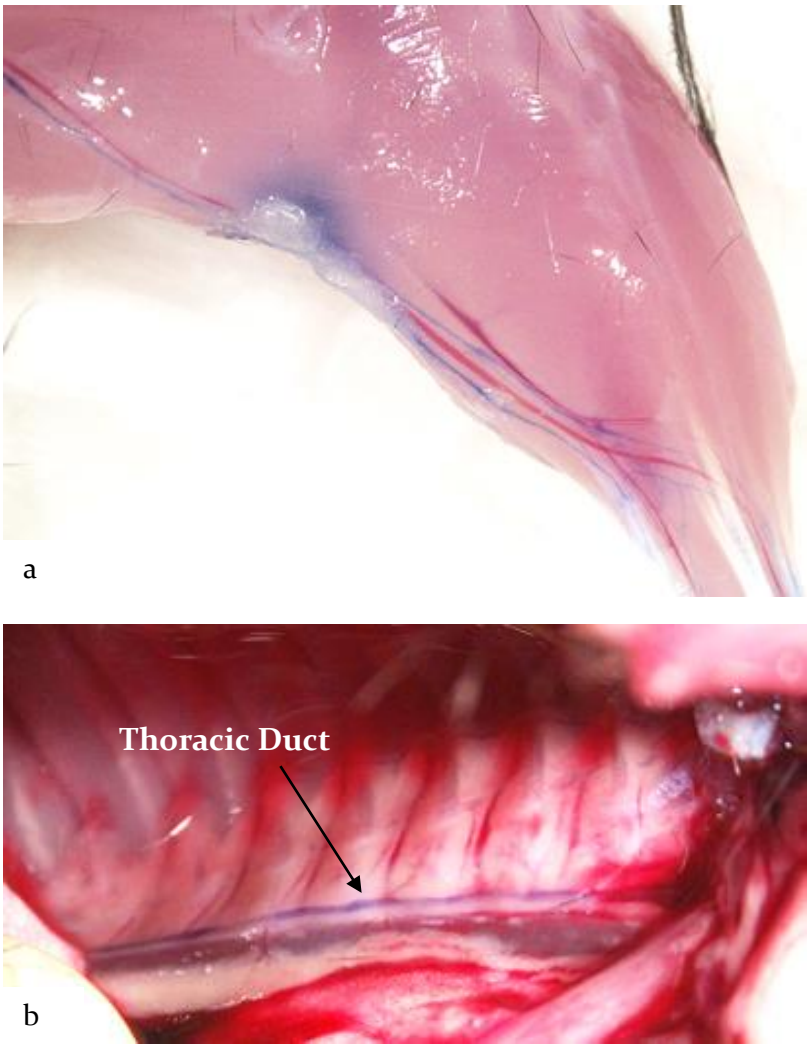

Figure 3-1: Evans Blue lymphatic vessel and popliteal lymph node right hind paw(a). Evans Blue in the thoracic duct (b).

3.4 Indocyanine green imaging in mice

A few recent studies have reported successful use of ICG in small animals to image real time lymphatic functioning after traumatic injuries or lymph node dissection, sentinel lymph node biopsies and cancer metastasis (9, 10). In the study by Burretta et al. (2013) imaging of the lymphatic system in pre- and post-surgical setting provide real-time imaging information of the lymphatic system response to surgery. Buretta et al. confirmed ICG is an effective, clinically translatable imaging modality for lymphatics (9). Proulx et al. bound ICG to lipids to increase its fluorescence intensity and administered this to vascular endothelial growth

factor (VEGF) -C overexpressed tumor bearing mice to image the lymphatic system with success (11). The ICG binding to lipids enhanced the fluorescence intensity and visibility of lymphatic vessels and nodes in deeper tissues. The mice images were analysed based on quantifying dynamic flow through signal intensity in the popliteal lymph node versus time, which was found to be high in tumor bearing mice compared to non-tumor-bearing mice (11).

By imaging the functioning of lymphatic vessels in *Gata2* mutant mice, we aimed to gain insight into features such as stagnating lymphatic flow, defective lymphatic vessel morphology and subclinical lymphoedema development that would increase our knowledge of the impact that structural defects of the lymphatic vasculature have on lymphatic vessel transport.

3.4.1 Objective

In collaboration with the Lymphatic Development Laboratory, I aimed to investigate lymphatic vessel transport in *Gata2* heterozygous compared to wild-type mice by:

1) Measuring the speed of ICG uptake up to the popliteal lymph node following footpad injection.

2) Developing a method to analyse lymphatic vessel pumping efficiency.

3.5 Methods: Lymphatic imaging method experiment one

Experiment one:

In this series of experiments, I used an IVIS® Lumina XRMS Series III Multi-species Optical and X-Ray Imaging System (PerkinElmer Health Sciences, USA) located at Adelaide Microscopy, University of Adelaide. This imaging device is used for *in vivo* bioluminescence and fluorescence imaging techniques and we sought to determine its usefulness of lymphatic vessel tracing with ICG.

All mice were housed at the SA Pathology Animal Care Facility, Adelaide. All experiments using mice were approved and conducted in accordance with the SA Pathology/CALHN Animal Ethics Committee and Australian National Health and Medical Research Council (NHMRC) guidelines. One *Gata2* heterozygous male and 2 litter mate controls, one male and one female, at 6 months of age were used for the first pilot of lymphatic imaging. One mouse at a time was anaesthetised with isoflurane in oxygen inhalation in an induction chamber and shaved of its fur of both hind limbs and iliac region, as dark fur reduces the detection of emitted fluorescence. Nair™ hair removal cream was then applied and washed off within 20 seconds with lukewarm water. The mouse was transported across the road to Adelaide University Microscopy Department according to GMO transportation guidelines.

IR-125 Laser Grade, Acros Organics Indocyanine green (Thermo Fisher Scientific, USA) was resuspended in sterile non-pyrogenic water at a concentration of 2.3mg/ml. This was allowed to dissolve by gently rotating the tube (foil-covered) for 20 minutes at room temperature. 10 microliters of reconstituted ICG was injected into both footpads with a 31 gauge syringe. The imaging was performed with the IVIS Lumina imaging system. The imaging parameters were set at default for ICG imaging. Briefly, this involved an excitation filter of 780nm, an emission filter 845nm and serial imaging with "no delay' meaning an exposure time of 0.5 seconds. Other settings were high sensitivity setting for the camera lens aperture (setting F=2) which controls amount of light and depth of field in order to obtain the optimum fluorescence intensity. A medium binning setting was selected by default which is the control of pixel size on the camera. We used a field of view set for 10cm X 10cm which would include visibility of the whole mouse excluding its tail. The region of interest was the popliteal lymph node of both hind paws. Following imaging of lymphatic transport through the popliteal node, the mice were humanely killed and their carcasses disposed of according to GMO guidelines.

Post image processing was performed with Living Image® Software 4.4.

3.6 Results: lymphatic imaging experiment one

The acquired images from the IVIS device showed effective transport of ICG fluorescence from the injection site into the popliteal lymph node in 2 out of the 3 imaged mice (Figure 3-2 a-b). The injection in the footpad was placed whilst the mouse was in the IVIS chamber. However, the delay between injection and starting the device before first measurement was between 30 seconds to 2 minutes causing unreliable data starting time points. Calculating the time versus ICG uptake up to popliteal lymph node would be unreliable given this delay.

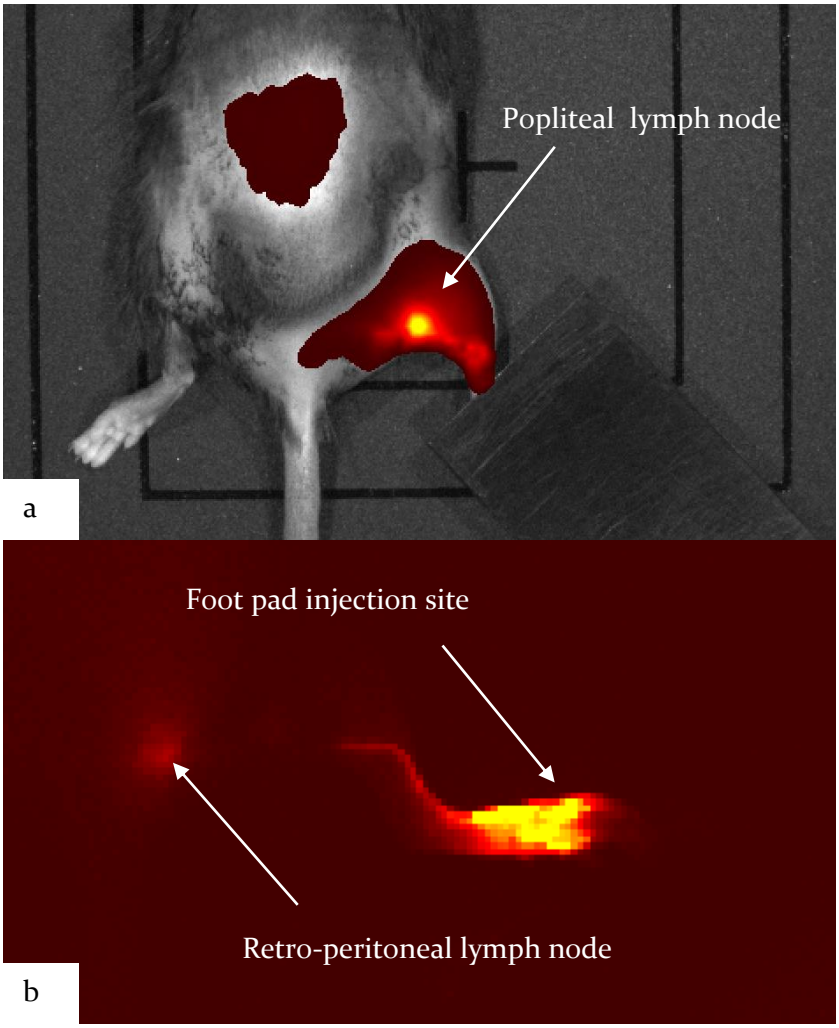

Figure 3-2: Position of the mouse in the IVIS and lymphatic transport of ICG from footpad to popliteal lymph node (a). Enlarged image with injection site, lymphatic vessel and peritoneal lymph node visible (b).

Post processing of images proved to be difficult as the resolution of the camera was not sufficient for adequate visualisation of lymphatic vessels in detail and in real-time.

To extract quantifiable data, such as photons per second and total radiance efficiency ($\mu W/cm^2$), a grid was superimposed on the images. Data acquired could not be compared with any other published lymphatic flow measurement due to the poor spatial resolution, and was deemed be unsuitable for analysis of lymphatic flow. Fluorescence intensity measurement on its own should be interpreted with care given any fluorescence backscatter or absorption could potentially result in false positive reading of forward flow or backward flow. Further imaging with the IVIS machine for this purpose of lymphatic vessel tracking and popliteal lymph node visualisation was therefore abandoned.

3.7 Methods: Lymphatic Imaging experiment two

A custom made near-infrared imaging camera (details *Chapter 2*) was modified to suit the needs for imaging small animals. Briefly it consisted of a security detection camera (CCD), with an 850 nm long-pass filter used to select the peak emission and avoid flooding of the detector. Eight excitation lasers of 780 nm wavelength each were angled on a custom made frame so they formed a homogenous excitation source (approximately 20cm from the object) (Figure 3-3).

The excitation lasers were switched on before recording to correctly position them on the mouse. Recording on a laptop with VirtualDub software started during the injection process and ambient light was switched off.

14 heterozygous mice (8 males and 6 females), and 9 litter mate controls (4 males and 5 females), 13 months of age, were used for lymphatic imaging and all experimental analyses were performed at the Animal Care Facility, SA Pathology, Adelaide. The appearance of ICG fluorescence in the popliteal lymph node was of interest including time from injection to the popliteal lymph node. This lymph node is anatomically fairly superficial and more likely to be strongly visible. Also the location did not require too much fur to be shaved off the mice.

This reduced the risk of hypothermia, overall deterioration whilst under anaesthetic and possibly an induced sluggish lymphatic flow.

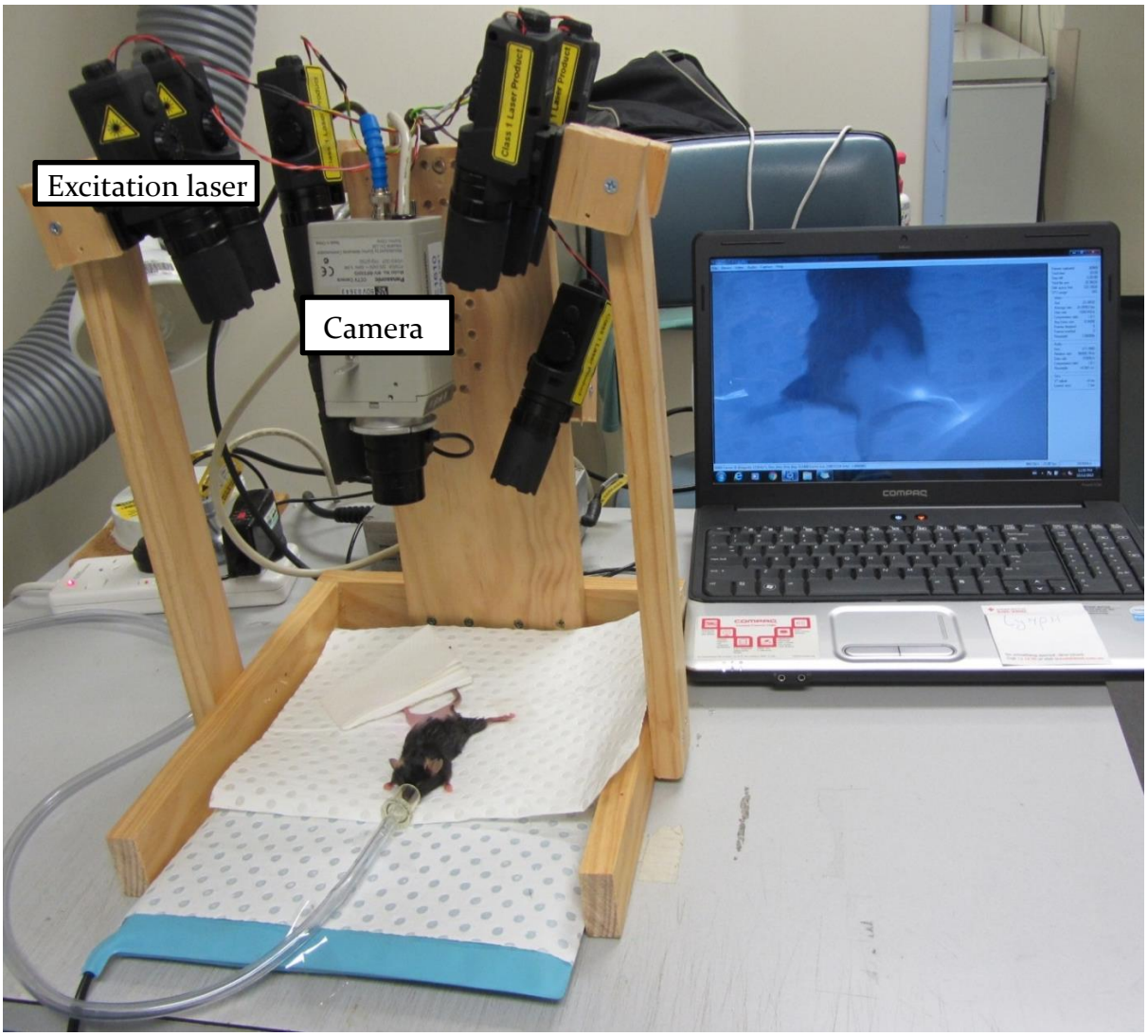

Figure 3-3: Experimental set up. Excitation class 1 lasers 780nm – 13mW output. CCD camera for capturing and laptop for recording with VirtualDub software.

A mouse at the time was anaesthetised with isoflurane inhalation anaesthetics in oxygen in an induction chamber and shaved of its fur of the right hind limb and iliac region. Nair™ hair removal cream was applied and washed off within 20 seconds with lukewarm water. The mouse was transported to a heat pad and placed on its ventral side with its nose in a nose cone with a continuous flow of isoflurane in oxygen. The mouse was placed in position for concentration of excitation light on the area of interest and within field of view of a custom made imaging camera system. Both hind limbs were taped to the underlying protection pad

with slight tension but no pressure on the foot and the footpad was slightly turned so it was facing up. Following imaging of lymphatic transport, the mice were humanely killed and their carcasses disposed of according to GMO guidelines.

IR-125 Laser Grade, Acros Organics Indocyanine green (Thermo Fisher Scientific, USA) was resuspended in sterile non-pyrogenic water at a concentration of 2.3mg/ml. This was allowed to dissolve by gently rotating the tube (foil-covered) for 20 minutes at room temperature. 10 microliters of reconstituted ICG was injected in the right footpad with a 31 gauge syringe. The injection site was covered with aluminium foil to minimise the fluorescence intensity bias of the injection site.

Videos of all mice were reviewed by 3 independent reviewers with the interest focussed on time from injection to visibility of the popliteal lymph node in seconds. The reviewers were blind to mouse id or details of mutation or wildtype. Data was transcribed in Excel and plotted in MATLAB.

A virtual stack of still TIF images for post processing was completed with ImageJ (1.49v, http://rsb.info.nih.gov/ij/,NIH, USA) and implementation with kymograph analysis that represents a dynamic process in a single image. Utilising the kymograph analysis approach has been published previously in other research settings where kymographic analysis of video sequences were used to form a spatio-temporal image (12, 13). The most visible lymphatic vessel linking to the popliteal lymph node was tracked with a 10 pixel segmented line (dotted yellow line (Figure 3-4 right). A kymograph slice was extracted and the line of the fluorescence intensity change was selected within a 10-pixel wide segmented line to obtain the lymph node relative saturation over time (Figure 3-4 bottom left). The diagonal white lines in the Kymograph slice representing vessel pulsation were also selected with a 10-pixel wide segmented line to obtain the lymph vessel pulsation (Figure 3-4 top left).

The values of the pulsation and saturation graphs were extracted and post processed with MATLAB Software 2015b. A MATLAB code was written to identify the slope and therefore the rate of uptake of ICG within the popliteal lymph node and to identify the most dominant peak in the vessel pulsation. The data was subjected to a fast Fourier transform, which decomposes a signal into frequencies, using Welch's power spectral density estimate to obtain the dominant pulsation frequency. A paired T-Test was applied to assess statistical significance between wildtype and *Gata2* heterozygous mice. The significance level was set at 5% ($\alpha = 0.05$).

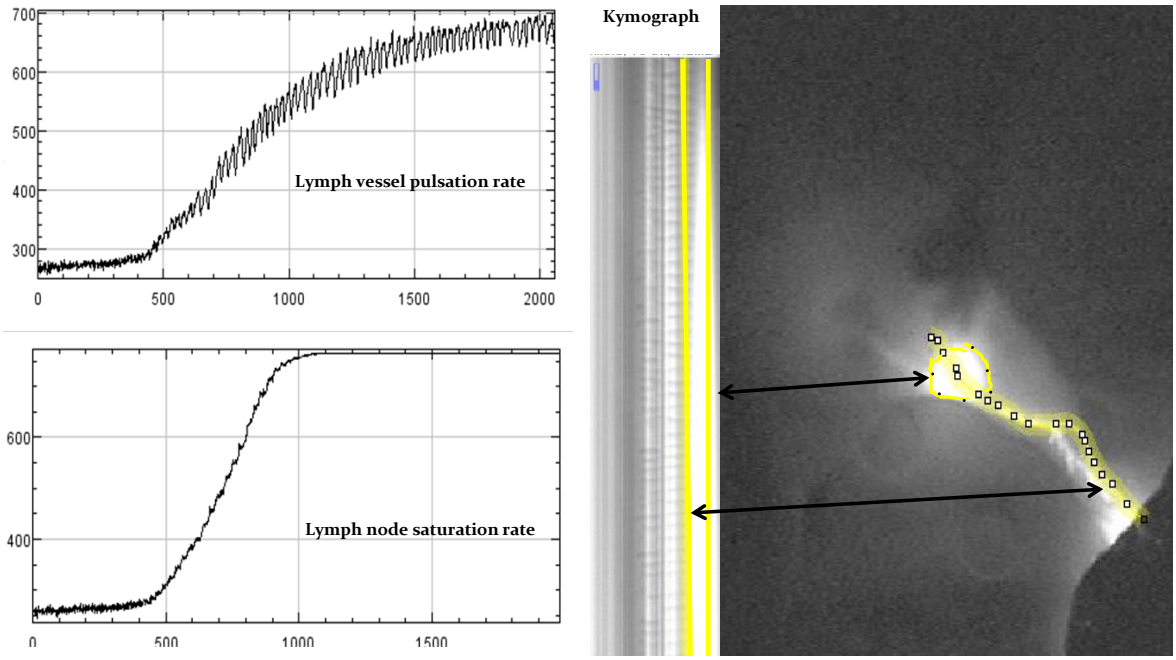

Figure 3-4: Kymograph analysis.

3.8 Results: Lymphatic imaging experiment two

In comparison to wildtype mice, *Gata2* heterozygous mice showed a variety of lymphatic vessel abnormalities. Abnormalities observed included enlarged popliteal lymph nodes, enlarged lymphatic vessels and lymphatic pooling (Figure 3-5). In addition, one *Gata2* heterozygous mice, out of 14, had no demonstrable ICG uptake in the popliteal lymph node.

Another *Gata2* mouse could not be analysed due to dark skin pigmentation in the popliteal node area. Two wildtype mice, out of 9, showed additional small lymph nodes in the hind limb close to the injection site.

Figure 3-5: a – Heterozygous mouse; arrow shows triangle shaped lymph node. b- Heterozygous mouse; arrow shows diffuse ICG pattern proximal from injection site. Note this is contained ICG and not back scatter as seen in image d. c- Heterozygous mouse; arrow shows enlarged lymph vessel. d- Wildtype mouse; arrow shows background scatter from intense ICG near the injection site, normal appearing lymph vessel. Note difference with image b. e- Wildtype mouse with normal appearing lymph vessels pattern. f- Wildtype mouse with normal appearing lymph vessels.

The three independent reviewers showed differences in the estimated time of ICG uptake and popliteal lymph node ICG fluorescence. Specifically, in mice id 124-127-132-134, all *Gata2* heterozygous mice, (Figure 3-6) it was seen that the reviewers have observational discrepancies of more than a minute. This could be explained by the comments given by the reviewers that the lymph vessels and lymph node was either “faint”, “blurry”, and/or “nondescript”.

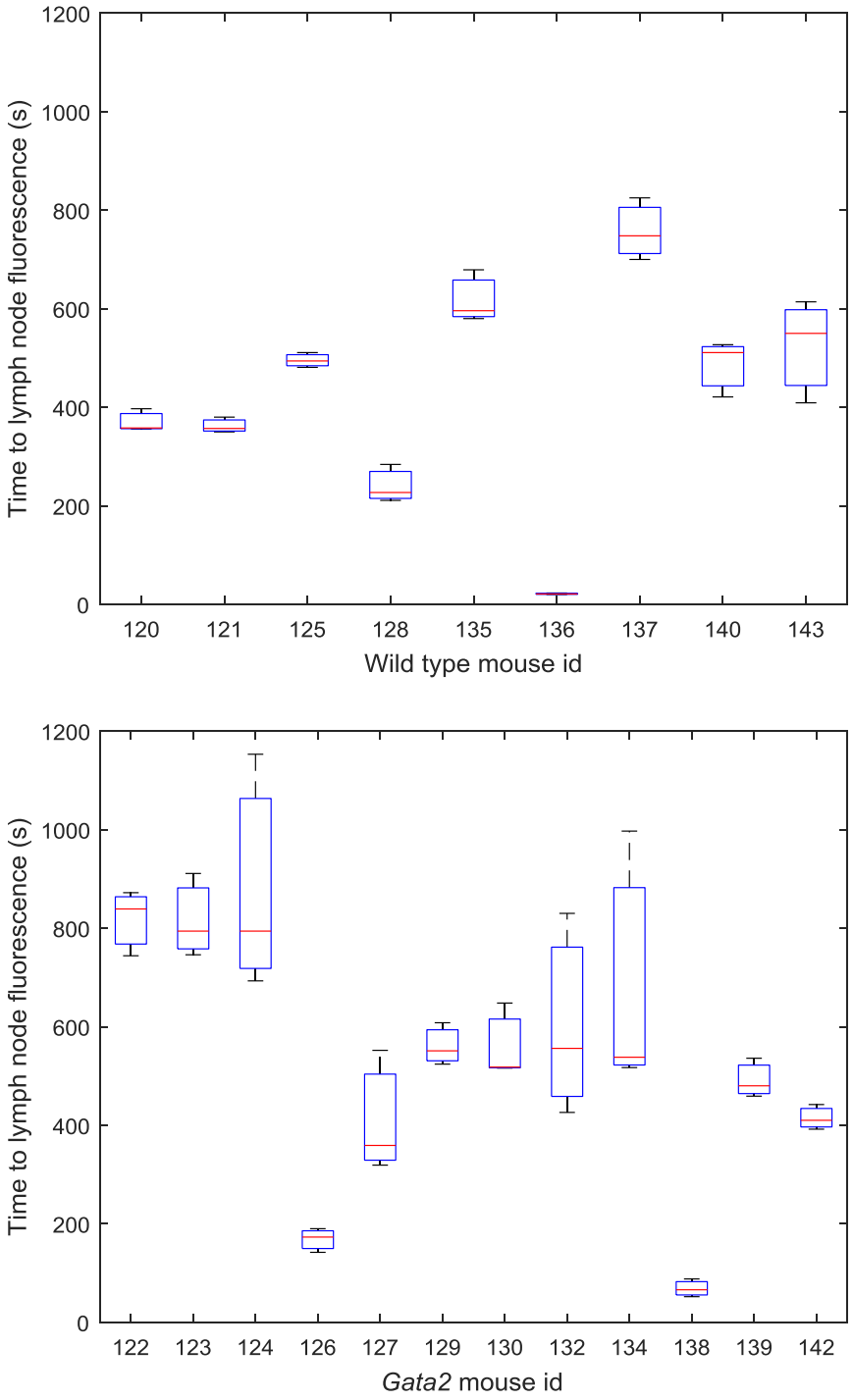

Figure 3-6: Three independent reviewers observation on time of popliteal lymph node appearing in wildtype (top)(n=9) and Gata2 mice (bottom) (n=12). Two Gata2 mice omitted due to no lymph node visible and dark skin pigmentation.

3.8.1 Results: Lymph node relative saturation

The rate of uptake, observed as an increase in pixel luminous intensity (fluorescence intensity) is normalised with minimum and maximum intensity, the value of 1 set as the maximum relative fluorescence intensity (Figure 3-7, 3-8). Two *Gata2* mutant mice did not show lymph node fluorescence and were therefore excluded from this analysis. This was due to very dark skin pigmentation on the area of popliteal node rather than no lymphatic uptake.

To smooth the data to reduce the background noise an 8th order polynomial has been fitted. Injections occurred whilst the recording started, however movement caused by injection, and the light needed to inject interfered with the start of the image caption. This caused uncertainty of determining the exact time of ICG injection, therefore the total time for a lymph node to become fully saturated could not be estimated accurately. However, by taking the derivative, the maximum saturation rate for each mouse can be determined as shown in figure 3.9 and 3.10. This may provide a good indication of the rate of influx into the lymph node.

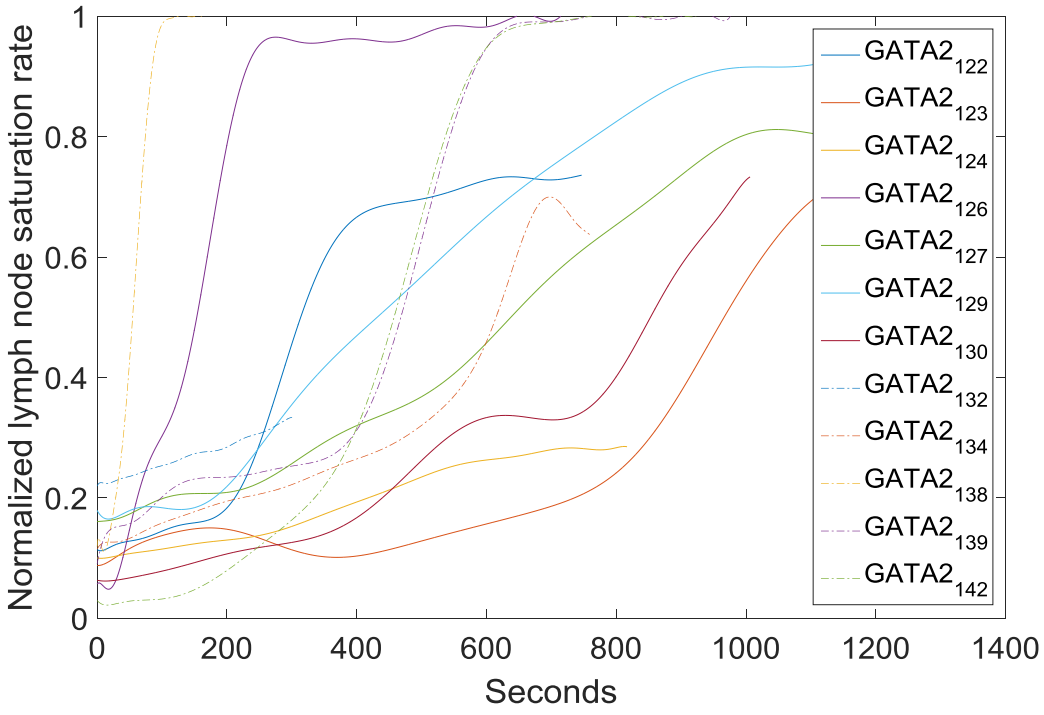

Figure 3-7: Normalised lymph node saturation rate for Gata2 mutant mice (n=12). Lines represent mouse id.

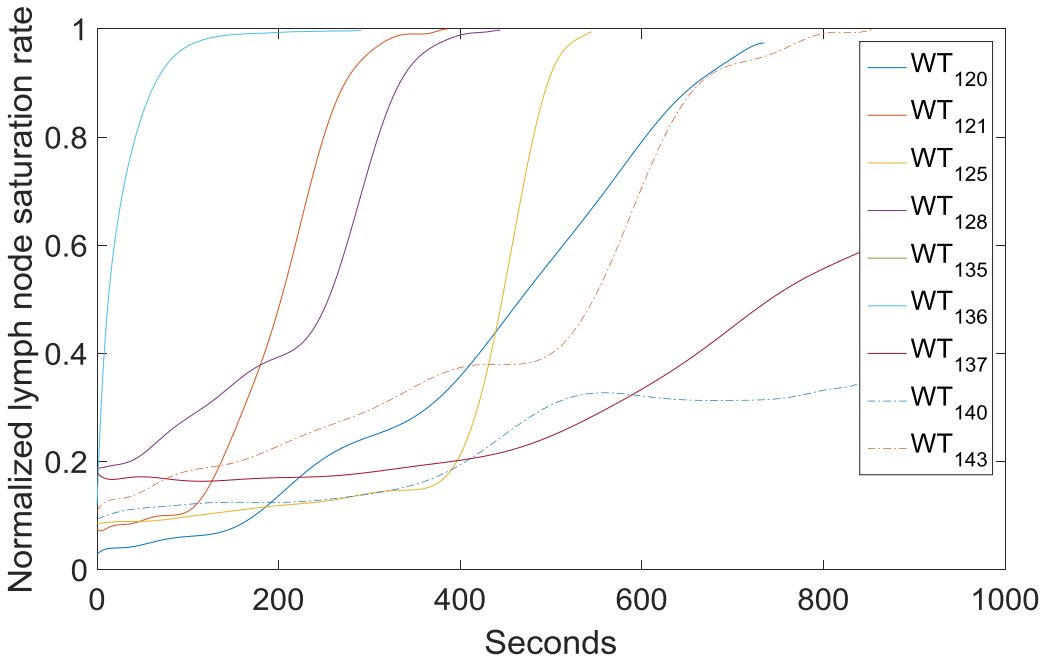

Figure 3-8: Normalised lymph node saturation rate for wildtype mice (n=9). Lines represent mouse id.

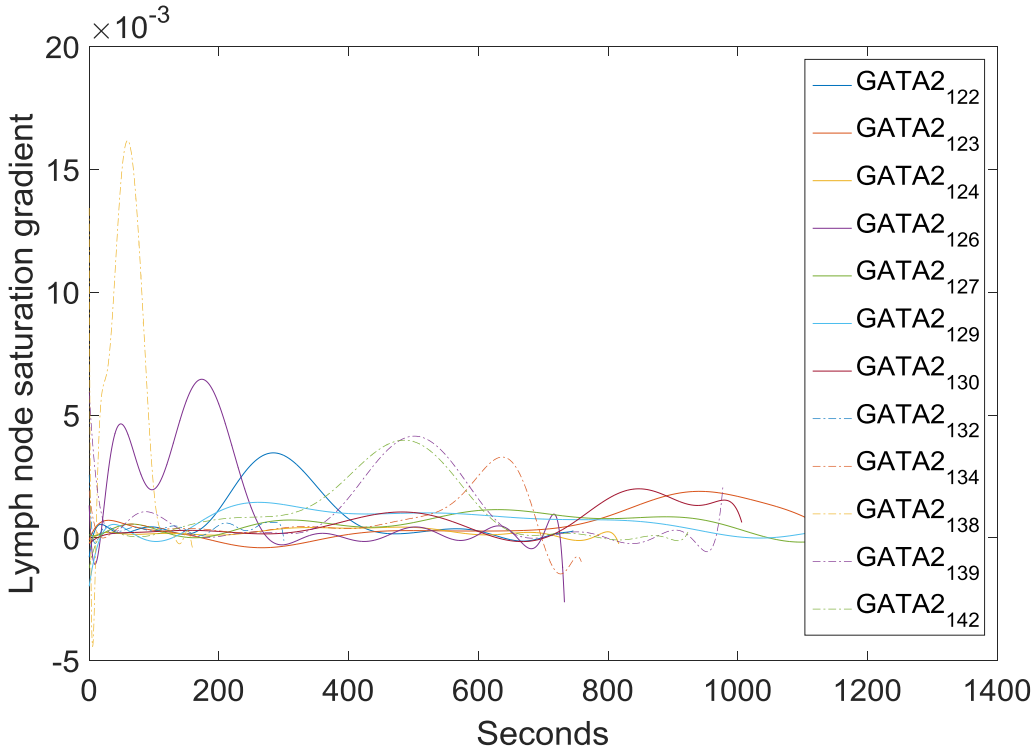

Figure 3-9: Lymph node saturation rate gradient for Gata2 mutant mice (n=12). Lines represent mouse id.

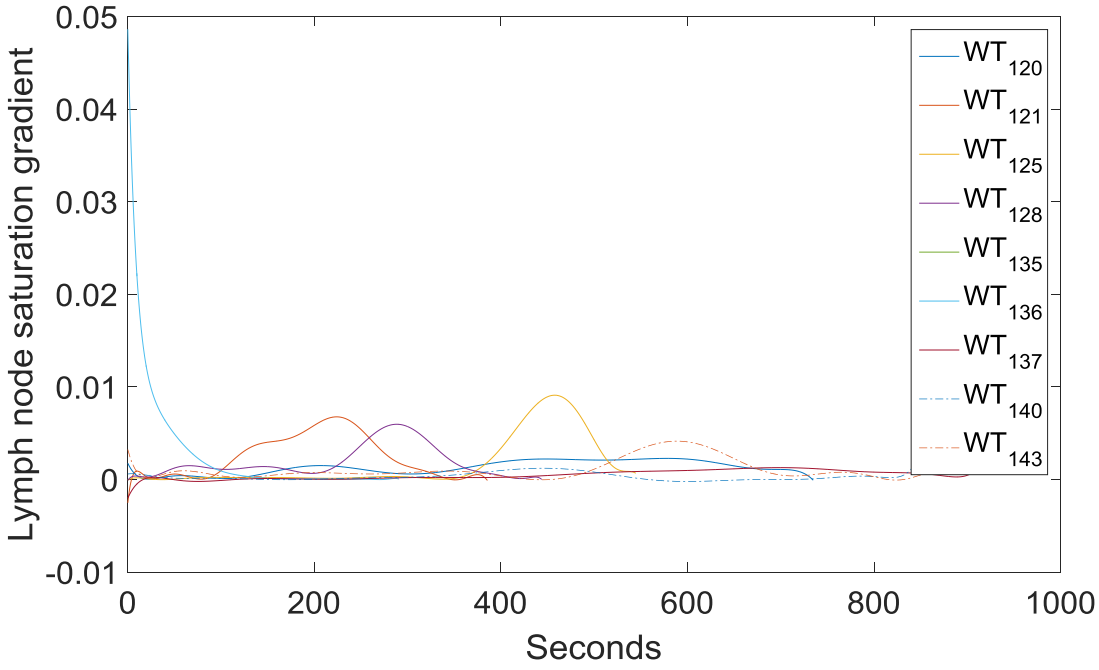

Figure 3-10: Lymph node saturation rate gradient for wildtype mice (n=9). Lines represent mouse id.

The data was subjected to a boxplot analysis and outliers outside of the 1.5 interquartile range (IQR) were omitted (14). Of the 12 *Gata2* heterozygous mice one outlier was omitted in the boxplot (n=11). Out of the 9 wildtype mice one outlier fell out of the 1.5 IQR and was also omitted (n=8). The boxplot (Figure 3-11) shows the maximum saturation rate was 61% faster in the wildtype compared to the *Gata2* heterozygous.

The median of the maximum relative saturation level of the popliteal lymph node in the *Gata2* heterozygous mice 0.002568 (IQR 0.00122), and wildtype median 0.0041471 (IQR 0.006551).

T-test data showed non-significant difference in lymph node rate of uptake between the *Gata2* heterozygous mice and wildtype mice (P = 0.207).

But there were observed abnormalities in the appearance of the lymph node, lymph pooling, and the lymphatic vessels in 8 of the 14 *Gata2* heterozygous mice with one of those unclear lymphatic uptake due to the dark skin pigmentation. One wildtype mice, out of 9, showed

extreme fast uptake of ICG in the lymph node, but overall all 9 wildtype mice appeared fairly consistent.

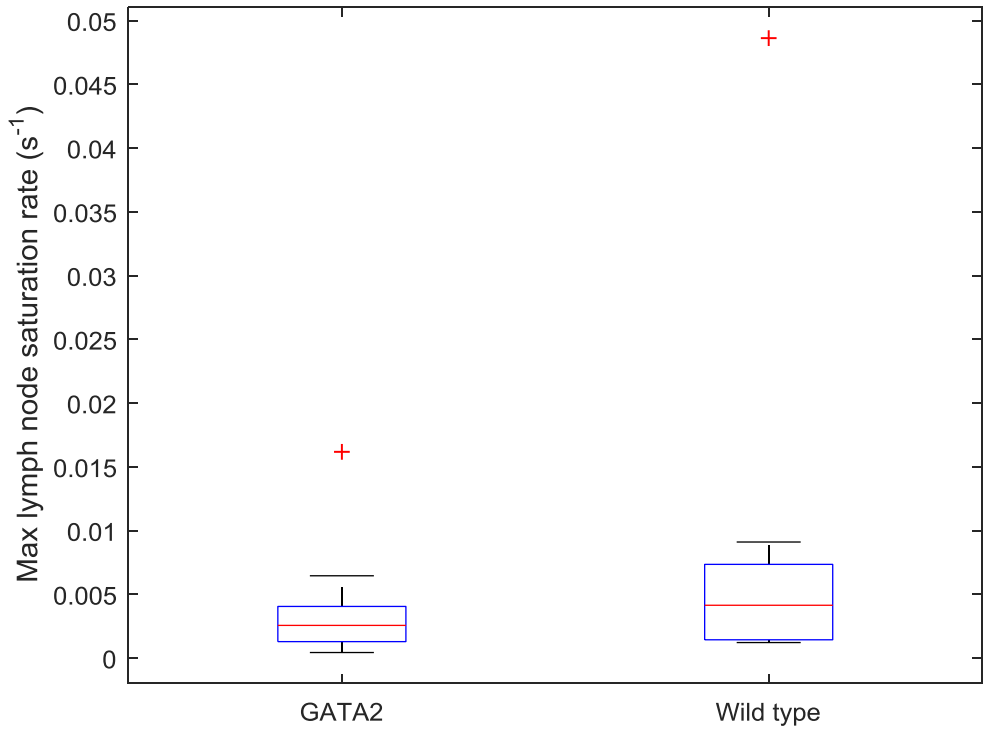

Figure 3-11: Boxplot median of max lymph node saturation rate of Gata2 mice omitted one outlier - id 138 (n=11), median 0.002568 (max 0.01618, min 0.00044). Wildtype mice omitted one outlier- id 136 (n=8) median 0.0041471 (max 0.048631, min 0.001222).

3.8.2 Results: Lymph vessel pulsation rate

The region of interest for detection of pulsation rate was the lymph vessel that linked from the injection site to the popliteal lymph node. The data has been subjected to a fast Fourier transform using Welch's power spectral density estimate to obtain the dominant pulsation frequency. Figure 3-12 shows a typical power spectral density of the most dominant peak (thus pulsation) that was extracted by using Welch's power spectral density estimate, and from this all dominant peaks were plotted (Figure 3-13).

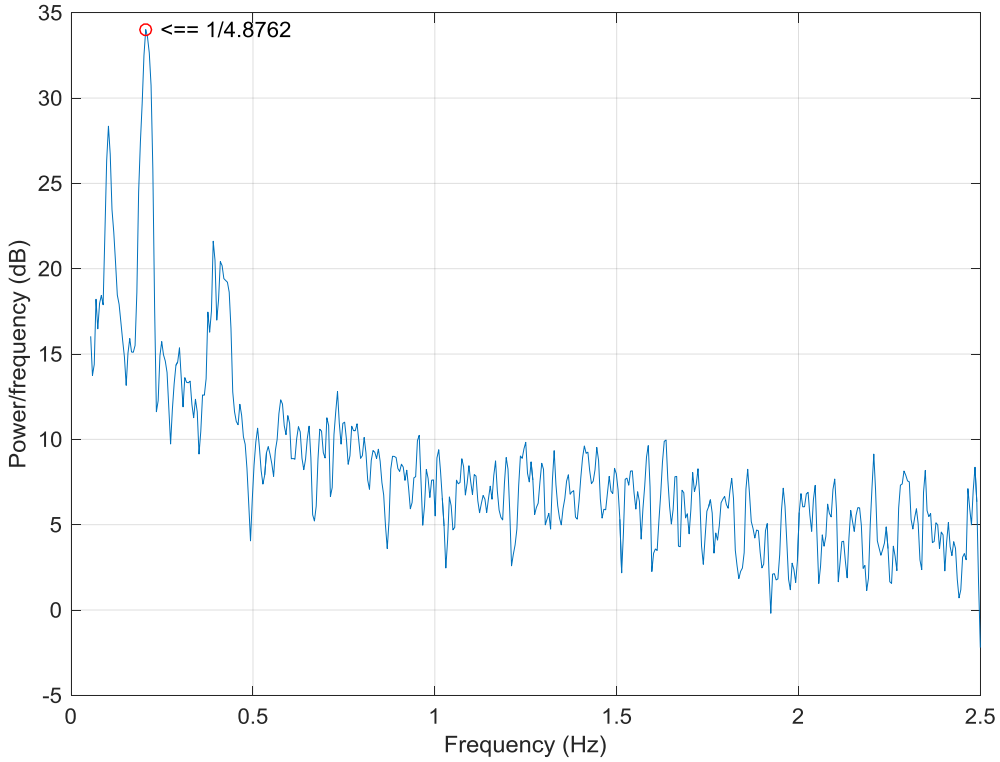

Figure 3-12: Example of power spectral density selecting most dominant pulsation.

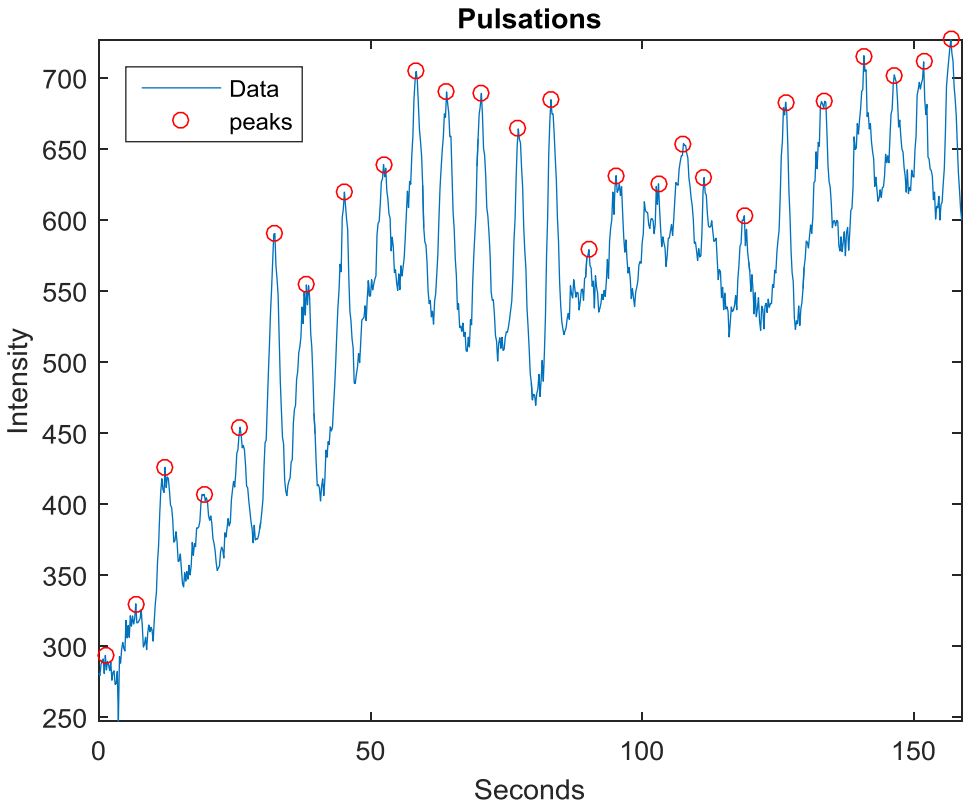

Figure 3-13: Peak finding for lymph vessel pulsation.

Under optimal anaesthesia the heart rate is steady between 300 and 450 beats per minute (~7 beats per second), and breath rate is set to 55 – 66 breaths per minute (~0.9 per second) (15). Therefore, since the dominant frequency was much less than this it could be concluded that the detected pulsation is indeed lymphatic vessel pulsation. All *Gata2* heterozygous mice could be included for the pulsation analysis (n=14), however two samples were omitted as they were outside of the 1.5 IQR range (n=12). The median pulsation rate was 0.15 beats per second in the *Gata2* heterozygous mice (n=12) and 0.20 beats per second in the wildtype (n=9) (Figure 3-14). No statistical significant difference in pulsation rate between the *Gata2* heterozygous mice and wildtype mice (t-test, P = 0.133) has been found.

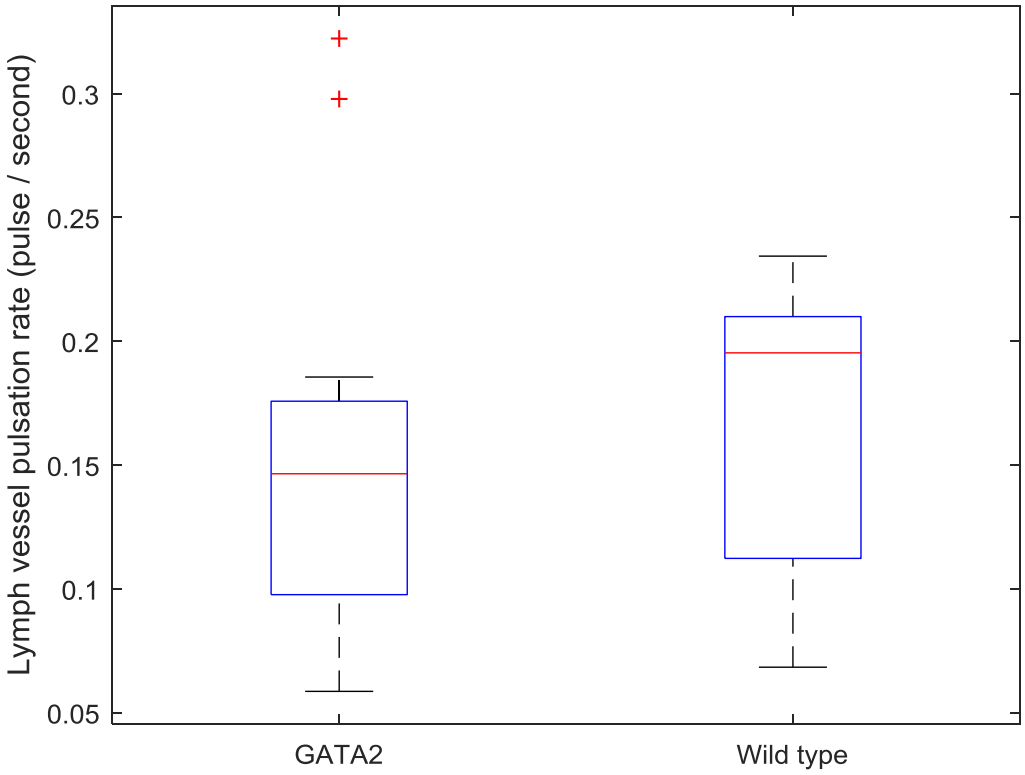

Figure 3-14: Boxplot median lymph vessel pulsation rate (seconds / pulse). Gata2 heterozygous two samples omitted (n=12) median 0.14648 (max 0.32227, min 0.058594). Wildtype (n=9) median 0.19531 (max 0.023437, min 0.068359).

3.9 Discussion and study limitations

In this pilot study, we found a way to visualise lymphatic vessel transport function in real time in *Gata2* heterozygous mice and their wild-type counterparts using ICG and near-infrared imaging. Lymphatic imaging in mice gives the researcher a more controlled and sensitive option for exploring lymphatic morphology and function in detail. Despite these advantages, fluorescence imaging with ICG or any type of fluorescence imaging is complicated to quantify. This is due to potential bias caused by scattering of the fluorescence emission, absorption and by camera saturation. However, previous attempts have been successful in quantifying frequency and velocity of ICG bolus movements and pulse rates over time in small animals (11, 16, 17).

Improved accuracy of detection of pulsation and potentially vessel diameter is still necessary to increase our understanding of lymphatic vessel function. A study recently published by Chong et al showed a technique with a focus on the lymphatic valves and contractility (18). Chong et al showed imaging of the actual lymphatic valves in real-time with a stereomicroscope camera. This is a technique we could replicate in the future with modification to our custom made imaging system.

It is known that introducing a tracer will likely change transmural pressure in the mouse and this should be noted or adjusted for in the final analysis (18). A limitation in this experiment was that we did not control for the exact quantity and exact pressure of administration of the ICG tracer. This could be prevented by introducing an automated infusion pump for the injections rather injecting by hand.

As is seen in the observational reviewers results in this chapter, the subjective value of time to fluorescence of the lymph node can vary drastically between observers and conclusions should be drawn from such results with absolute care. But, the observed abnormalities in the G*ata2* heterozygous mice compared to the wildtype mice showed varieties. Four *Gata2*

heterozygous mice showing "faint" appearing of the lymph node (Figure 3-6), one reported without ICG uptake at all, and another three with visible abnormalities such as enlarged popliteal lymph nodes, enlarged lymphatic vessels and lymphatic pooling (Figure 3-5).However, this did not appear to affect lymphatic transit time or pulsation rate, since there was no significant difference in lymph node saturation time or pulsation rate in the *Gata2* heterozygous mice versus the wildtype mice. This data was rather variable, perhaps due to the biologically variance in the mice, methodological limitations or a combination of these.

Designing a computer generated algorithm to analyse fluorescence data from time to injection to area of interest would be more valuable than observational or subjective data. Such fluorescence analysis could potentially be with a kymograph approach which has been used for quantifying small moving objects in biological research such as in the field of neurology (12). Exploring and implementing kymograph analysis showed that such analysis is feasible in small laboratory animals.

The lack of difference in measured lymphatic function between the wildtype and heterozygous mice is consistent with the physical appearance of the fairly aged mice (~13 months old), which showed no visible oedema. *Gata2* heterozygous mice did seem to have more adipose tissue which may indicate a dysfunction in the lymphatic system leading to adipogenesis (19, 20).

These experiments have demonstrated that ICG imaging gives a good indication of lymphatic functioning in real-time in mice.

3.10 Chapter conclusions

Real-time lymphatic imaging with ICG in *Gata2* heterozygous mice is an effective and informative measurement to enhance our understanding of the consequences of gene mutation on lymphatic flow and function.

References

1. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. Cell. 1999;98(6):769-78.

2. Sabin FR. On the origin of the lymphatic system from the veins and the development of the lymph hearts and thoracic duct in the pig. American Joural of Anatomy. 1902.

3. Harvey NL. Lymphatic vascular development. Heart Development and Regeneration 2010. p. 543-65.

4. Tammela T, Alitalo K. Lymphangiogenesis: Molecular mechanisms and future promise. Cell. 2010;140(4):460-76.

5. Oliver G, Srinivasan RS. Endothelial cell plasticity: how to become and remain a lymphatic endothelial cell. Development. 2010;137(3):363-72.

6. Gordon K, Ostergaard P. Impact of genetics on lymphangiogenesis. In: P C Neligan, Masia J, Piller NB, editors. Lymphedema Complete Medical and Surgical Management. 1. USA: Taylore & Francis Group; 2015. p. 121-35.

7. Kazenwadel J, Secker GA, Liu YJ, Rosenfeld JA, Wildin RS, Cuellar-Rodriguez J, et al. Loss-of-function germline GATA2 mutations in patients with MDS/AML or MonoMAC syndrome and primary lymphedema reveal a key role for GATA2 in the lymphatic vasculature. Blood. 2012;119(5):1283-91.

8. Ostergaard P, Simpson MA, Connell FC, Steward CG, Brice G, Woollard WJ, et al. Mutations in GATA2 cause primary lymphedema associated with a predisposition to acute myeloid leukemia (Emberger syndrome). Nature genetics. 2011;43(10):929-31.

9. Buretta KJ, Brat GA, Christensen JM, Ibrahim Z, Grahammer J, Furtmüller GJ, et al. Near-infrared lymphography as a minimally invasive modality for imaging lymphatic reconstitution in a rat orthotopic hind limb transplantation model. Transplant International. 2013;26(9):928-37.

10. Blum KS, Proulx ST, Luciani P, Leroux J-C, Detmar M. Dynamics of lymphatic regeneration and flow patterns after lymph node dissection. Breast Cancer Res Treat Breast Cancer Research and Treatment. 2013;139(1):81-6.

11. Proulx ST, Luciani P, Derzsi S, Rinderknecht M, Mumprecht V, Leroux JC, et al. Quantitative imaging of lymphatic function with liposomal indocyanine green. Journal of Cancer Research. 2010;70(18):7053-62.

12. Mukherjee A, Jenkins B, Fang C, Radke RJ, Banker G, Roysam B. Automated kymograph analysis for profiling axonal transport of secretory granules. Medical Image Analysis. 2011;15(3):354-67.

13. ImageJ. Generate and Exploit Kymographs: ImageJ; 2016 [updated 20/05/2016. Available from: http://imagej.net/Generate_and_exploit_Kymographs.

14. Boslaugh S. Statistics in a Nutshell. 2012;2nd Edition.

15. Ewald AJ, Werb Z, Egeblad M. Monitoring of vital signs for long-term survival of mice under anesthesia. Cold Spring Harbor Protocols. 2011;6(2).

16. Weiler M, Kassis T, Dixon JB. Sensitivity analysis of near-infrared functional lymphatic imaging. Journal of Biomedical Optics. 2012;17(6):1-11.

17. Proulx ST, Luciani P, Christiansen A, Karaman S, Blum KS, Rinderknecht M, et al. Use of a PEG-conjugated bright near-infrared dye for functional imaging of rerouting of tumor lymphatic drainage after sentinel lymph node metastasis. Journal of Biomaterials. 2013;34(21):5128-37.

18. Chong C, Scholkmann F, Bachmann SB, Luciani P, Leroux JC, Detmar M, et al. In vivo visualization and quantification of collecting lymphatic vessel contractility using near-infrared imaging. Scientific reports. 2016;6.

19. Harvey NL. The Link between Lymphatic Function and Adipose Biology. Annals of the New York Academy of Sciences. 2008;1131(01):82-8.

20. Harvey NL, Srinivasan RS, Dillard ME, Johnson NC, Witte MH, Boyd K, et al. Lymphatic vascular defects promoted by Prox1 haploinsufficiency cause adult-onset obesity. Nature genetics. 2005;37(10):1072-81.

CHAPTER 4
Human research materials and methods

To improve our understanding of this peripheral lymph system and its response to injury, a trial was conducted with lower limb trauma patients. The lymph flow around the injured and reconstructed site, was monitored, together with the effect of the injury on the patients wellbeing. The custom made Indocyanine green based lymphatic imaging system, described in *Chapter 2: Imaging the lymphatic system,* was a core component of this trial. This chapter describes the materials and methods used to conduct the trial

4.1 Materials and method of application

The assessment equipment and study survey materials described in this thesis have been used in multiple research protocols and studies conducted within the Lymphoedema Clinical Research Unit in the Department of Surgery at Flinders University, South Australia. All equipment has been internationally validated with the tools acknowledged as oedema assessment tools by the International Society of Lymphology (Arizona, USA) (1).

The lymphatic imaging camera was designed and built in conjunction with the Biomedical Engineering Department at Flinders Medical Centre. The design of the camera was based on the imaging requirements of the study and has been validated for:

- Correct excitation wavelength and intensity
- Correct fluorescence wavelength and intensity
- Distance of camera versus object
- Penetration depth of imaging
- Optimal field of view.

These parameters were compared to the technical specifications for indocyanine green (ICG) lymphography as described in the literature (2-5).

The following sections describe the equipment, the survey instruments, together with their use and application in the studies presented in this thesis.

4.2 Patient surveys and demographic data collection

The following surveys were administered to gain an understanding of each individual's background, medical history related to the trauma, general health and functioning of the lower limb after the trauma.

- *General patient medical history form*

A form was created with questions related to the nature of the trauma, when it occurred, any pain they were experiencing and, if so, where and its severity, any medication, and any physiotherapy or other treatments related to the trauma. The form also contained questions about the individual's general socio-demographic background (Appendix B). General patient information was obtained from the database kept at the Plastic and Reconstructive Surgery Department, Royal Adelaide Hospital, South Australia. The nature of the injury, surgical intervention, infection and any complications were noted on a generic patient medical history form from this database. The medical notes were consulted when additional information was needed, such as the duration of vacuum-assisted closure (VAC), the type of antibiotics administered or the details of a complication. The medical notes were requested with assistance from the Department's administrative staff and a clinical plastic surgery registrar co-investigator. Upon enrolment in the study, participants were asked specific questions related to their injury according to the medical history form (Appendix B). Additionally, the participant's height, weight, blood pressure and pulse were also recorded.

- *General Health Short Survey Form (SF-12)*

The General Health Short Survey Form (SF-12) is used in a variety of settings to measure the health status of individuals or a population. The SF-12 is a self-rated and self-administered general health questionnaire consisting of 12 questions. The SF-12 has been implemented and validated nationally and internationally (6-9). However, only a few outcome studies have been published that have applied the SF-12 specifically for lower limb trauma patients (10-13). One such study by Patel et al. has shown the successful administration of SF-12 to a cohort of 36 patients who had undergone reconstructive surgery of their lower extremity (13).

The questionnaire has two components: the Mental Health Component Summary (MCS) score and the Physical Health Component Summary (PCS) score. A licence was obtained from QualityMetric™ to use their SF-12 version 1 and QualityMetric Health Outcomes™ Scoring Software 4.0 (Appendix C).

The questions focused on the general health status of the participant. The questions were based on how the participant felt over the previous four weeks, and were specifically based on general physical performance and also on general mental performance. This questionnaire was self-administered on hard copy and later transcribed into Excel format for further processing and scoring with the QualityMetric Scoring Software. STATA 12.1 was used for statistical T-Test.

- *Lower Extremity Functional Scale (LEFS)*

The Lower Extremity Functional Scale (LEFS) is a questionnaire that is designed to focus on the functioning of the lower extremities after they have experienced a traumatic or invasive event(s). The questionnaire is self-administered and consists of 20 questions. This questionnaire has been validated and has also been tested for sensitivity to change compared to the General Health Questionnaire (SF-36) and has been deemed superior (14, 15). In this 20-item questionnaire, the questions focused on the participant's lower limb functioning.

The questionnaire has been successfully applied in previous studies of lower limb trauma population. The questions relate to movement restriction in the lower limb following the trauma and subsequent reconstruction. Questions cover most aspects of specific movement that are part of daily living. This questionnaire was self-administered on a hard copy (Appendix D) and later transcribed onto an Excel spreadsheet.

4.2.1 Patient assessment

To assess each patient's general physical details, photographic images of the study and control leg, patient's height, weight and the dimensions of the reconstructed area were collected.

- *Clinical photos*

Digital images were taken in 2014 with a Canon SX230 HS compact camera to establish the baseline measures. In 2015, the clinical photos were taken with a digital single lens Canon EOS camera. Photos of the lower limb were taken from four different angles with the participant standing upright when possible. The sides photographed were: anterior both legs, lateral side left, lateral side right and posterior both legs.

- *Height and Weight Watchers® Conair™ weight scale*

Height was measured in centimetres with a measuring tape fixed to the wall within the research space. Weight was measured in kilograms on a 3V DC battery-operated weight scale (Weight Watchers® Conair™ model WW60A).

- *Size of the flap*

The length and width of the reconstructed site were measured in centimetres (1–180 cm) with a BSN Medical JOBST® generic measurement tape.

The tape was applied across the reconstructed area to measure the size of the reconstructed site (the scar area). The measurements recorded were from distal to proximal (in cm), from medial to lateral (in cm), and diagonal (in cm).

4.2.2 Fluid measurement

The following tools were used to obtain accurate details on the whole limb segmental fluids and site-specific fluids, limb volumes and skin integrity.

- *IMP™ SFB7 Bioimpedance Spectroscopy (ImpediMed Ltd, Queensland, Australia)*

Impedance measurements have been used to determine total body water (TBW) since the 1970s. Bioimpedance has been validated to assess unilateral oedema with almost 100% specificity and sensitivity (16). The IMP™ bioimpedance portable measurement tool SFB7 uses a single-channel, tetra-polar bioimpedance spectroscopy (BIS) approach. It scans 256 frequencies between 4 kHz and 1000 kHz. The device utilises Cole-Cole modelling with the Hanai mixture theory to determine TBW, extracellular fluid (ECF) and intracellular fluid (ICF) from impedance data. The fat-free mass (FFM), fat mass (FM) and Body Mass Index (BMI) are then calculated based on standard algorithms (17). Disposable pre-gelled electrocardiogram (EKG)-style electrodes (ImpediMed Ltd, Queensland, Australia) were used to acquire a reading. The electrodes were affixed to the hand, wrist, ankle and foot (using standard anatomical locations) and the leads of the device were attached for the measurement of each segment. The patient remained in a supine position on a non-conductive hospital bed during all measurements.

The measured impedance in ohm (indicated by Z), is related to the volume of conductive fluid, total body water (TBW), within the body according to the following relationship:

$$TBW = \rho \frac{L2}{Z} \tag{4.1}$$

Where ρ is the specific resistivity of body fluids (ohm.cm), L is the distance between electrodes and Z is the measured impedance or resistance (ohm). Consequently, a change in the magnitude of Z is inversely proportional change in the volume of TBW. Impedance of body's tissues is dependent upon the frequency (Hz) of the applied electric current such that at low frequency, ideally zero, the current cannot pass across cell membranes which act as biological electrical capacitors and, hence, the change in impedance is now inversely proportional to the change in extracellular water volume (ECW):

$$ECW = \rho \frac{L2}{Z_{low}} \tag{4.2}$$

Were Z_{low} is the impedance measured at a low frequency. Impedance is a vector quantity comprising two components: reactance (Xc), the opposition to current flow due to the capacitive nature of cell membranes and resistance (R), the opposition to current flow due to the inherent resistance of tissue fluids:

$$ECW = \rho \frac{L2}{R_{low}} \tag{4.3}$$

where R_{low} is the resistance of the extracellular fluid. Lymph is an extracellular fluid; lymphoedema, a condition in which lymph fluid accumulates in tissues, elicits an increase in ECW that is measured as a decrease in R_{low} according to equation 4.3 (18).

Research software, Impedimed Whole Body Impedance Processing Software version 5.2.2.0, was used for data recording, storage and processing. A 12-month follow-up measurement was taken, where all segmental measurements were repeated three times and an extra measurement was added to obtain specific fluid data of the area below the knee. This was an experimental measurement to investigate the presence of extracellular fluid within the reconstructed area only. This required the placement of one extra disposable electrode under the knee. For accuracy of the measurement outcome, the participant's weight was recorded

with a standard weighing scale and height was recorded in centimetres with a measurement tape attached to the wall (Paragraph 4.2.1).

- *Moisture Meter D® (Delfin Technology Limited, Kuopio, Finland)*

The Moisture Meter D® is a small portable battery-powered measurement device that measures the tissue dielectric constant (TDC) via a probe that touches the skin surface (19). The medium probe used for this study is reported to have a penetration depth of 2.5 mm. The TDC reading can be converted to relative local tissue water (LTW) (20) with the following equation:

$$\mathrm{LTW\%} = \frac{100(\mathrm{TDC} - 1)}{77.5} \tag{4.4}$$

The Moisture Meter D® is validated and has a high inter-observability agreement (21). A higher TDC value and thus higher relative local tissue water is suggesting a higher fluid content between the probe on the skin and the superficial fascia.

DelWin 4 software was used to store the repeated data during measurements, and to extract the mean and export it to Microsoft Excel 2010. Further statistical analysis (linear mixed effect model) was performed with Statistical Software STATA 12.1.

The measurement was conducted in the middle of the flap as determined by observation with the same region on the other leg selected as a control measurement (Figure 4-1). Initially, the dorsal surface of the feet were included in the measurement protocol. However, in most cases, the probe was unable to be completely sealed on the skin and therefore those readings would not be reliable. This measurement location was therefore abandoned.

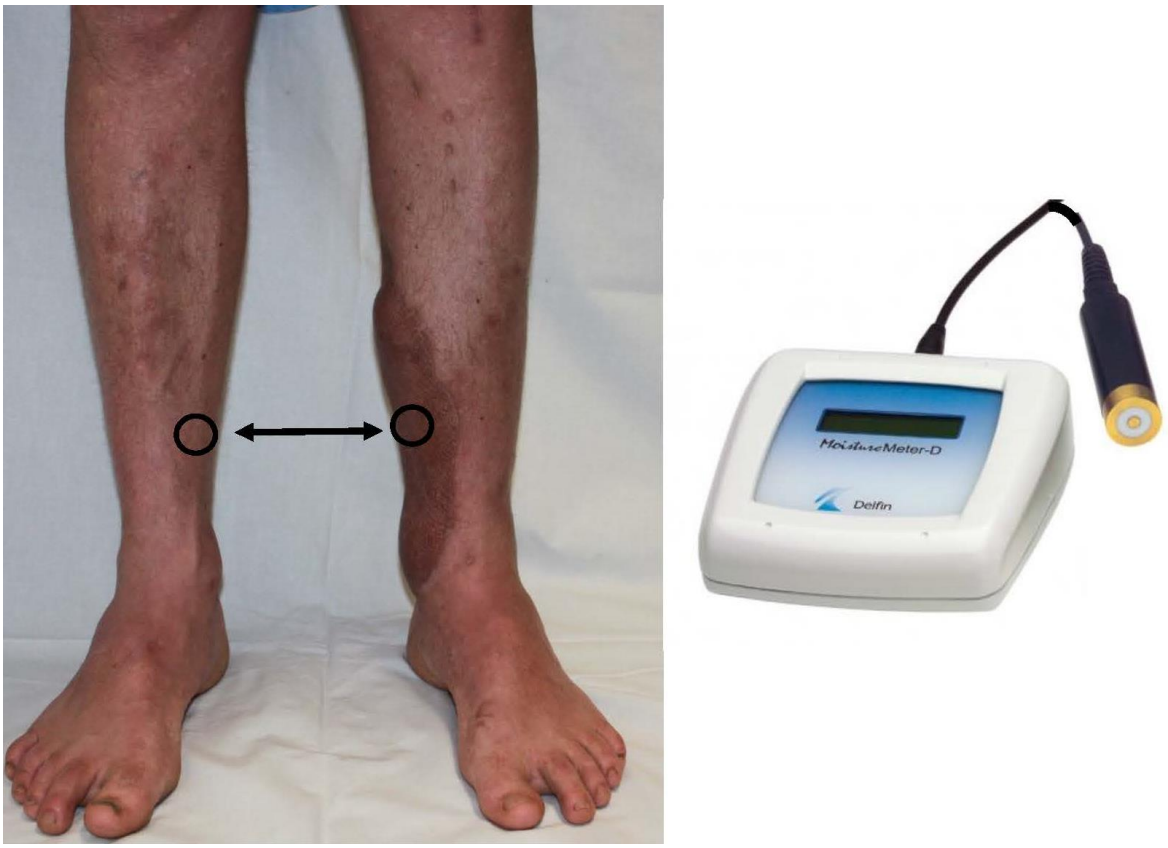

Figure 4-1: Placement of the Moisture Meter probe.

- *Vapometer® (Delfin Technology Limited, Kuopio, Finland)*

The Vapometer® is a hand-held, battery-operated, closed-chamber trans-epidermal water loss (TEWL) measurement tool. It measures the flux of water vapour diffusing through the skin. This water vapour is captured by a cylindrically shaped chamber that is placed on the skin and contains a Honeywell humidity sensor (HIH 3605-B) and a temperature probe. The device needs to be in contact with the skin surface to obtain a measurement. With the device in contact with the skin surface (an area of ~1.5 cm^2), the skin will seal the chamber that contains the measurement probes. The Vapometer® measures the skin's relative humidity and ambient temperature within the sealed chamber (22). The term 'relative humidity' means "the amount of water vapour present in air expressed as a percentage of the amount needed for saturation at the same temperature" (23). The flux of water vapour, which represents TEWL, is expressed as $g/m^2/h^1$ (the coefficient of capillary suction) of water. The

measurement of TEWL provides information about the skin surface and skin barrier function: more water loss or increased water-evaporation loss indicates damage to the skin resulting in a loss of its barrier function. The Vapometer® is reliable and has been validated and correlated along with other TEWL devices with open and closed chambers (24, 25).

As with the Moisture Meter D®, DelWin 4 software was used to store the repeated data during measurements, and to extract the mean and export it to Microsoft Excel 2010. Further analysis for repeatability and statistically significance was performed with Statistical Software STATA 12.1 (96) (Paragraph 4.3.4).

The measurement was conducted in the middle of the flap as determined by observation with the same region on the other leg selected as a control measurement. Initially, the dorsal surface of the feet was included in the measurement protocol; however, in most cases as with the Moisture Meter, the probe was unable to touch the skin which resulted in highly variable data or extremely low readings. This is consistent with other research which mentioned that this could be due to the thickness of the skin on the dorsal side of the foot

- *Tape measurement*

A BSN Medical JOBST® generic measurement tape (1–180 cm tape) was used. for circumference measurement of both legs at 4 cm intervals. Marks were set on the lateral side of the leg starting at 10 cm from the heel (in approximately the anatomical location of the lateral malleoli). For more accuracy in detecting smaller differences, measurement intervals of 4 cm were adopted up to 74 cm maximum. The obtained circumferential data were processed with a truncated cone formula (Formula 4.5).

The application of a measurement tape is a common way in which to determine the increase of size of a specific area. It has been argued that it is not sensitive enough in determining small changes and that it is susceptible to measurement errors but it is a user-friendly and quick measurement. The Australasian Lymphology Association (ALA) has included this basic

circumference measurement technique in their national guidelines to standardise measurement techniques: this technique is also included in the international consensus document in regard to tape circumference measurement (1, 26, 27). The techniques described in these documents are as follows:

- Apply measurement board under the leg;
- Start measurement point at the malleoli as the anatomical reference (this correlates with approximately 10 cm from heel to ankle);
- From this starting point measure and mark every 10 cm until the top of the leg is reached.

The participant was in supine position for circumference measurements, and volume was calculated in Excel with the truncated cone formula:

$$V = \frac{1}{3}\pi h(R^2 + r^2 + Rr) \tag{4.5}$$

were *V* is volume, *R* is major radius and *r* is minor radius.

4.2.3 Lymphatic imaging

A contrast agent and an imaging system were used to obtain information about lymphatic vessel location and functioning:

- *Contrast agent: indocyanine green (ICG) (Pulsion Medical Systems)*

Indocyanine green (ICG) is a water-soluble tricarbocyanine dye which has been used clinically for over 50 years for the imaging of cardiac output, hepatic function and retinal vascularisation (28). Now it is used to visualise the architecture and functioning of lymphatic vessels, ICG is recognised as being less invasive and an accurate lymph visualisation technique (4). After injection into biological tissues, ICG immediately binds to plasma proteins and the dye molecules are excited (~850 nm) from their ground state by tissue penetration of a near-infrared excitation light (~780 nm). It is then captured by a camera that is sensitive to near-

infrared imaging and that contains an ~830 nm filter between the lens and the camera unit. This filter is necessary to isolate the ICG fluorescence and avoid flooding of the image by the excitation light source of ~780 nm. Indocyanine green (ICG) has a low toxicity and it is entirely processed and excreted by the liver (29). No adverse events have been reported with the use of ICG for lymphatic imaging (4, 28, 30, 31).

However, ICG is not generally approved by the Therapeutic Goods Administration (TGA) in Australia for administering in lymphatic imaging. An 'authorised prescriber' is allowed to prescribe and order the clinical test with ICG for a specific patient or it can be used within the boundaries of a clinical trial under a Clinical Trial Notification (CTN) scheme (trial number 2013/0049 protocol 121123).

- *Near-infrared imaging camera*

The camera that was used to acquire ICG images was custom modified by the Biomedical Engineering Department at Flinders Medical Centre and Flinders University. Custom-made cameras for the purpose of imaging the superficial lymphatic vessels with ICG have proven useful as reflected in previously published studies. The specifications required to non-invasively image the superficial lymphatic vessels by means of ICG were available and seemed relatively easy to replicate (2, 5).

Our custom-made apparatus consisted of:

- a digital charge-coupled device (CCD) camera (Panasonic, WV-BP330) (32).
- an aspherical infrared (IR) varifocal lens (Model number VIR 3080AS: Daiwon Optical, Korea) (33).

- an 850 nm longpass filter (Edmund Optics, Singapore) mounted behind the camera lens to ensure ICG emission spectra isolation by filtering out wavelengths below 850 nm to prevent flooding of the image (34).

- eight battery-operated Pulsar L-808S laser-diode flashlights (780 nm wavelength) (Pulsar, USA) attached to a mounting ring around the camera and acting as the excitation light source. This laser-diode is classified as a Class 1 laser which is safe in all conditions as the maximum permissible exposure cannot be exceeded (35).

Indocyanine green (ICG) fluorescence has a unique absorption and emission spectrum in the near infrared (NIR) range. By using ICG fluorescence and the detectors in the NIR range, the vessels of interest can be visualised up to approximately 1.5 cm through the skin.

The camera and laptop were tested to, and passed, Australian/New Zealand Standard (AS/NZS) 3551:2004 "Technical management programs for medical devices", as Class 1 BF equipment.

The indocyanine green (ICG) was obtained from Pulsion Medical Systems, Germany. One vial of 25 mg ICG was reconstituted with 5 mL water for injection. An amount of 0.1 mL per injection was administered intradermally twice with a 0.5 mL insulin syringe and a 30 gauge needle. The location of the two intradermal injections was into the dorsal space of both feet at the base of the toes near the first and fourth web space. The participants were offered an anaesthetic cream (EMLA, LMX4) to reduce the discomfort of the injection itself. The dermis, up to the epidermis, contains many nerve endings and any intradermal injection can be painful. The participant was in a supine position on an examination couch for the injection. After the injection, the participant remained in a supine position but was requested to wiggle their toes and move their ankle (if capable) to activate the calf muscle pump. In cases of slow lymphatic flow or limited uptake of ICG, visible as a light and diffuse pattern of lymphatic

vessels via the imaging system, the participant was asked to walk around within the research space (approximately 10 m^2). The investigator in some cases needed to enhance the flow of lymph in the skin with light pressure on the injection site to assist the uptake of ICG, followed by stroking the dorsal side of the foot if needed up to the ankle region. Care was taken with these minor interventions as ICG is very easy to contaminate in the surrounding tissues and might cause fluorescence that is not related to the functioning of lymphatic vessels.

In four selected cases with large muscle free flaps, ICG was injected within the flap or 5 mm distal from the scar tissue border. These selected cases are clearly identified in the results in Chapter 5

For the initial imaging, the fluorescence in the lymph vessels was visualised in real time by the custom-made camera system (see Section 4.2.3). The camera was placed approximately 50 cm from the leg for the best focus.

For all follow-up data, the commercially available near-infrared camera, Hamamatsu Photo Dynamic Eye (Hamamatsu, Japan), was available for usage. Fluorescence images were directly comparable between devices with the small hand-held Hamamatsu camera being more user-friendly.

- *Hamamatsu Photo Dynamic Eye (PDE Hamamatsu, Japan)*

The Hamamatsu Photo Dynamic Eye is a commercially available near-infrared (NIR) camera with a charge-coupled device (CCD) and an LED excitation light source. The LED light source does not require specific safety measures for the research participant or researcher (36). Since June 2014, it has been a TGA-approved device (Medical Device Class 1) supplied by SDR Scientific Pty Ltd, NSW.

- *Imaging processing and storage*

All videos were recorded in real time with recording software (VirtualDub version1.14.10) on a CompaQ Presario CQ60 laptop (37). Uncompressed AVI video format and VirtualDub were used for post-recording processing, such as selecting still images. Freeware software ImageJ (version 1.49) was used to enhance images by reducing noise (38). The Java-run Lymphatic Vessel Analysis Protocol (LVAP) plug-in was used to count lymph branches, loops and lymphatic vessel density. Back-ups of all measurements were saved on a 1 terabyte (TB)-Passport external hard drive.

4.3 Recruitment method

Patients were recruited from an existing lower limb trauma database kept by the Department of Plastic and Reconstructive Surgery at the Royal Adelaide Hospital, South Australia. Patients on this database had undergone reconstructive surgery on the lower extremity related to high-impact mechanical trauma between 2009 and 2015. This included patients who had suffered an injury on their lower limbs involving a car, motorbike or other motorised vehicle, or who had experienced falls from a height (>4 metres), attack from livestock or work-related accidents. The extent of their injury and its location required surgical reconstruction in order to salvage the limb. The reconstructive surgery included soft tissue reconstruction such as free muscle flaps for large defects with bone exposure or local tissue flaps for smaller and less complex defects. Free muscle flaps are technically demanding for the performing surgeon and are often only implemented with a trauma case that has significant soft tissue loss with a high risk of infection, bone exposure and a need for a reconstruction with a sufficient blood supply. Local tissue flaps often involve rotation of soft tissue to cover the defect without detaching the flap's blood supply or a local flap can be a muscle that is fitted into the defect (e.g. the gastrocnemius muscle to cover a tibial defect). The database also includes patients who have received split thickness skin grafts or full thickness skin grafts that were used to cover a defect related to lower limb injury. The

database contains information about each individual's trauma details, surgical intervention, infection and adverse events as well as basic socio-demographic data. This ongoing database is kept and regularly updated by the Department of Plastic and Reconstructive Surgery.

After consulting the OACIS Clinical Information System / electronic health records on correct contact details, deceased status and residential address within South Australia, 86 patients received an information package via post. This package consisted of a letter of invitation signed by the Head of the Department of Plastic and Reconstructive Surgery; a detailed patient information sheet; a consent form; and an opt-out leaflet with a reply-paid envelope. All patients were informed in the patient information sheet that a follow-up phone call may occur two weeks after being posted the information package. Patients could opt out over the phone or, if the information had not been received at the time of the phone call, a new information package would be sent out.

Recruitment from the Plastic and Reconstructive Surgery inpatient ward occurred when the principal investigator and thesis author was notified by the medical, administration or nursing staff of a new patient who had received reconstructive surgery related to lower limb trauma. These patients received information about the study verbally as well as receiving hard copies of the letter of introduction, participant information sheet, consent form and the opt-out leaflet. The patient was invited to consider participating in the study and to discuss this with a family member or a friend. The anticipated measurement of the patients in this aspect of the study was set at the first follow-up appointment (approximately six weeks' post-reconstructive surgery). Given the early nature of the appointment after surgery and their inclusion in a research project, a patient could only be enrolled after the appropriate clearance from the treating surgical staff member.

4.3.1 Inclusion criteria

The following criteria had to be met for the patient to be included in the study:

- The patient had undergone bone (orthopaedic surgery) and soft tissue (plastic surgery) reconstruction for severe compound fractures hereinafter described as the 'surgical intervention': 'severity of compound fractures' was as defined per the Gustilo open fracture classification system;
- Surgical intervention had occurred between January 2009 and August 2015;
- The patient was over the age of 18;
- The patient had the ability to give informed consent based on their mental competence and ability to make their own decisions.

4.3.2 Exclusion criteria

Patients were excluded if :

- They were under the age of 18;
- They were unable to give written consent;
- They were not being followed up in the Royal Adelaide Hospital Plastic and Reconstructive Surgery outpatient department. Generally, this meant that the person had moved interstate or overseas or had been imprisoned;
- They required amputation (either as part of their immediate treatment or subsequently due to complications);
- They were deceased (state-wide hospital records were checked prior to attempted contact through the Oacis Hospital Management Information System [HMIS]);
- They had a known or perceived reaction to the contrast agent indocyanine green (ICG) or to contrast agents in general or had an allergy to iodides;
- They were pregnant.

Follow-up

To measure any change over time, the cohort of patients who participated in the study in 2014 were invited by phone to return for a 12-month follow-up measurement at the Royal

Adelaide Hospital Department of Plastic and Reconstructive Surgery. Participants were reminded of the protocol with a verbal explanation or, if requested, the participant information sheet was sent via post or email (the latter if requested specifically by the participant).

Participants had the opportunity to verbally opt out of the ICG imaging measurement but still could participate in the remaining measurements.

All the measurements that were performed in 2014, as indicated earlier in this chapter, were repeated in the same order and under the same conditions.

4.3.3 Additional information

The protocol stated that if persistent local or general leg swelling (oedema or lymphoedema) was identified, the participant needed to be referred back to the treating physician for further diagnostic tests or management. Three participants were identified with significant swelling and ongoing problems relating to lymphoedema. These cases were discussed with the treating specialist and a referral for a diagnostic test (a lymphoscintigram) was organised by staff of the Plastic and Reconstructive Surgery Department. It was verbally explained to participants that this diagnostic test itself was not part of the research project. However, results of the test provided additional, valuable information regarding their deeper lymphatic vessels and therefore participants were asked for their approval to use their scan results in the research results. The participants receiving further medical care signed an additional patient information sheet and consent form to have their data included in any reports, publications or scientific meetings.

4.3.4 Statistical analysis

All data was transcribed in Excel database and imported into STATA 12.1 for further statistical analyses. All data was checked for normality using histogram distribution and a fitted curve. The statistical significance level was set at 5% ($\alpha = 0.05$).

Descriptive statistics was used to compare the study participants with the complete lower limb database (Chapter 5.2). Also descriptive statistics was used on reporting circumferential measurements and it is calculated volume with the truncated cone formula (Formula 4.2), the Indocyanine Green lymphatic imaging, and the general patient medical history.

Inferential statistics were used to test the significance of differences between the affected or reconstructed limbs and the contra-lateral control limbs. This was tested at baseline and at the 12 month follow up.

- IMP™ SFB7 Bioimpedance Spectroscopy (ImpediMed Ltd, Queensland, Australia).

Descriptive statistics of the patient demographics was used next to a paired T-Test to test the significant difference of the mean extra-cellular fluid (R_0) in the affected limb versus the contra-lateral control limb. The T-Test would be most appropriate in testing pairs that are spatial and to analyse statistically meaningful difference between the two sample means (affected and control leg respectively).

All measurements at baseline were single measurements and the measurements at follow up were the average of 3 repeated measurements.

- Moisture Meter D® (Delfin Technology Limited, Kuopio, Finland)

The values of the Moisture Meter are expressed in tissue di-electric constants or TDC values. A more meaningful way is expressing the values in local tissue water (LTW) when applying formula 4.4. All Moisture Meter mean TDC data points (3 repeated measurements) were adjusted for the individuals BMI (assuming no change of coefficients). The data of affected

limb was compared to the contra-lateral control limb and tested for statistically significant difference with the linear mixed effect model. This model was chosen for random effect and fixed effects in the data. As fixed effect we selected the participant ID and BMI as both are expected to be an effect. With this model we could explore a wide variety of correlation patterns. The quantitative outcome is the mean score of the 3 repeated measurements within subject ID.

- Vapometer® (Delfin Technology Limited, Kuipio, Finland)

The Vapometer data was subjected to a covariance of variance analysis to examine for the reliability of measurement before further analysis was made. If reliability was met a linear mixed effect model with ID and age as fixed effect would be used for determination of statistically significance.

- General health questionnaire (SF-12)

The mean difference of the mental component summary (MCS) and the physical component summary (PCS) at baseline was compared to the mean MCS and mean PCS at follow-up. A paired T-Test was chosen to be suitable to compare the two sample means between baseline and follow up.

- Lower extremity functioning scale (LEFS)

The mean difference of the lower extremity functioning scale (LEFS) at baseline was compared to the mean LEFS at follow-up. A paired T-Test was chosen to be suitable to compare the two sample means between baseline and follow up.

References

1. International Society of Lymphology. The diagnosis and treatment of peripheral lymphedema: 2013 Consensus Document of the International Society of Lymphology. Lymphology. 2013;46(1):1-11.

2. Kitai T, Inomoto T, Miwa M, Shikayama T. Fluorescence navigation with indocyanine green for detecting sentinel lymph nodes in breast cancer. International Journal of Breast Cancer. 2005;12(3):211-5.

3. Sharma R, Wendt JA, Rasmussen JC, Adams KE, Marshall MV, Sevick-Muraca EM. New horizons for imaging lymphatic function. Annals of the New York Academy of Sciences. 2008;1131(01):13-36.

4. Marshall MV, Rasmussen JC, Tan IC, Aldrich MB, Adams KE, Wang X, et al. Near-Infrared fluorescence imaging in humans with indocyanine green: A review and update. The Open Surgical Oncology Journal. 2010;2:12-25.

5. Weiler M, Kassis T, Dixon JB. Sensitivity analysis of near-infrared functional lymphatic imaging. Journal of Biomedical Optics. 2012;17(6):1-11.

6. Ware J, Kosinski M, Keller S. A 12-Item Short-Form Health Survey: cunstruction of scales and preliminary tests of reliability and validity. Medical Care. 1996;34(3):220-33.

7. Farivar SS, Cunningham WE, Hays RD. Correlated physical and mental health summary scores for the SF-36 and SF-12 Health Survey, V.1. Health Qual Life Outcomes Health and Quality of Life Outcomes. 2007;5(1):1-8.

8. Sanderson K, Andrews G. The SF-12 in the Australian population: cross-validation of item selection. Australian and New Zealand journal of public health. 2002;26(4):343-5.

9. Tucker G, Adams R, Wilson D. New Australian population scoring coefficients for the old version of the SF-36 and SF-12 health status questionnaires. International Journal of Quality of Life Aspects of Treatment, Care and Rehabilitation. 2010;19(7):1069-76.

10. Kiely M, Weidner K, Weigelt J. Correlation of SF-36 and SF-12 in a trauma population. Journal of Surgical Research Journal of Surgical Research. 2006;130(2):227-8.

11. Bedigrew KM, Patzkowski JC, Wilken JM, Owens JG, Blanck RV, Stinner DJ, et al. Can an Integrated Orthotic and Rehabilitation Program Decrease Pain and Improve Function After Lower Extremity Trauma? Journal of Clinical Orthopaedics Related Research. 2014;472(10):3017-25.

12. Tay W-H, de Steiger R, Richardson M, Gruen R, Balogh ZJ. Health outcomes of delayed union and nonunion of femoral and tibial shaft fractures. International Journal of Care of the Injured. 2014;45(10):1653-8.

13. Patel KM, Economides JM, Franklin B, Sosin M, Attinger C, Ducic I. Correlating patient-reported outcomes and ambulation success following microsurgical lower extremity reconstruction in comorbid patients. Journal of Microsurgery. 2014;34(1):1-4.

14. Lin CW, Moseley AM, Refshauge KM, Bundy AC. The lower extremity functional scale has good clinimetric properties in people with ankle fracture. Physical therapy. 2009;89(6):580-8.

15. Binkley JM, Lott SA, Riddle DL, Stratford PW. The Lower Extremity Functional Scale (LEFS): Scale Development, Measurement Properties, and Clinical Application. Physical therapy. 1999 1999/04//:371.

16. Cornish B, Chapman M, Hirst C, Mirolo B, Bunce I, Ward L, et al. Early diagnosis of lymphedema using multiple frequency bioimpedance Lymphology. 2001;34:2-11.

17. Cornish BH, Jacobs A, Thomas BJ, Ward LC. Optimizing electrode sites for segmental bioimpedance measurements. Physiological measurement. 1999;20(3):241-50.

18. Van Zanten M, Piller NB, Ward L. Inter-changeability of Impedance Devices for lymphedema Assessment. Journal of Lymphatic Research and Biology. 2015.

19. Aimoto A, Matsumoto T. Noninvasive method for measuring the electrical properties of deep tissues using an open-ended coaxial probe. Medical Engineering & Physics. 1996;18(8):641-6.

20. Mayrovitz H, Weingrad D, Brlit F, Lopez L, Desfor R. Tissue dielectric constant (TDC) as an index of localized arm skin water: differences between measuring probes and genders. Lymphology. 2015;48(1):15-23.

21. Mayrovitz H, Bernal M, Brlit F, Desfor R. Biophysical measures of skin tissue water: variations within and among anatomical sites and correlations between measures. Skin Research and Technology. 2013;19:47-54.

22. Tagami H, Kobayashi H, Kikuchi K. A portable device using a closed chamber system for measuring transepidermal water loss: comparison with the conventional method. Skin Research and Technology. 2002;8(1):7-12.

23. 2015. Online Dictionary.

24. De Paepe K, Houben E, Adam R, Wiesemann F, Rogiers V. Validation of the VapoMeter, a closed unventilated chamber system to assess transepidermal water loss vs. the open chamber Tewameters. Skin Research and Technology. 2005;11:61-9.

25. Imhof RE, De Jesus ME, Xiao P, Ciortea LI, Berg EP. Closed-chamber transepidermal water loss measurement: microclimate, calibration and performance. International journal of cosmetic science. 2009;31(2):97-118.

26. Australasian Lymphology Association. Guideline for a national standard technique of measurement of lymphoedematous limbs. ALA, 2004.

27. Lymphoedema Framework. Best Practice for the Management of Lymphoedema. International consensus. London: Medical Education Partnership Ltd, 2006.

28. Suami H, Chang DW, Yamada K, Kimata Y. Use of indocyanine green fluorescent lymphography for evaluating dynamic lymphatic status. Plastic and reconstructive surgery. 2011;127(3).

29. PULSION-Medical-Systems. Summary of product characteristics, specifications sheet. Munich, Germany: PULSION Medical Systems; 2007.

30. Sevick-Muraca EM. Translation of near-infrared fluorescence imaging technologies: emerging clinical applications. Annu Rev Med. 2012;63:217-31.

31. Sevick-Muraca E, Kwon S, Rasmussen J. Emerging lymphatic imaging technologies for mouse and man. Journal of Clinical Investigation. 2014;124(3):905-14.

32. Panasonic. Panasonic Digial Singal Processing 1/3" B/W CCD Cameras WV-BP330 series Specification. In: Company P-VIS, editor. Panasonic Canada Inc.

33. Daiwon. Aspherical IR Carifocal Lens Owner's Manual. In: Ltd DOC, editor.

34. Edmund Optics. Emund Optics; [Available from: http://www.edmundoptics.com/optics/optical-filters/longpass-edge-filters/high-performance-od-4-longpass-filters/66228.

35. PULSAR. Pulsar L-808S/L-915 Laser IR Flashlight Instructions.

36. PDE Photodynamic Eye Handbook [press release]. 2010.

37. VirtualDub video capture/processing utilit [Internet]. Avery Lee. 2013.

38. Rasband W. ImageJ Software USA2015 [Available from: http://imageJ.nih.gov/ij.

CHAPTER 5
Human research results

This *Chapter* will provide the results obtained in the human trial that took place at the Royal Adelaide hospital (RAH), Department of Plastic and Reconstructive Surgery. In summary, investigating the effect of lower leg injury on the functioning of the lymphatic system and patient wellbeing, revealed that whilst acute injuries generally healed well, lymphatic system regeneration was poor and patient functioning was low. Tissue reconstruction sites were seen to have little or no functional superficial lymphatic vessels.

5.1 Background

The Department of Plastic and Reconstructive Surgery treated over 90 patients between 2009 and 2014 with lower limb soft tissue reconstruction. 86 patients of these were identified with Gustilo IIIB compound fracture and were included in a comprehensive database. Demographic data of 83 of these patients was extracted from medical notes by clinical research staff (recruitment database, Table 5-1). 23 out of 83 agreed to take part (Table 5-1). The flowchart of the recruitment process is outlined in Figure 5-1.

5.2 Results: comparing study participants with overall lower limb trauma recruitment database.

This lower limb trauma recruitment database of 83 patients consisted of 82% (n=68) males and 18% (n=15) females, with the mean (±SE) age of 47 (±2.1) years (range 20 to 97). Fracture of the left leg was slightly more common than the right leg with (53%, n=44) with the mid tibia fracture being the most common site of injury (39%, n=32). 35% (n=29) of lower limb trauma was associated with falls, followed by motorbike accidents (MBA) (30%, n=25), motor vehicle accidents (MVA) (23%, n=19) and lastly crush injury (12%, n=10).

Table 5-1 Description of study participants (n=23) and the recruitment database (n= 83) ± Standard Error. Fracture lower limb; most common fractures, Adverse event; most common adverse event, Outcome upon discharge; bone union at 3 month follow up (3/12), Mean time; relevant time related data in days (D) and hours (H), Soft tissue reconstruction; vacuum assisted closure (VAC) in days (D).

Description	Details	Study participants 23	Recruitment database 83
Gender	Male / female	19 (83%) / 4 (17%)	68 (82%) / 15(18%)
	Mean age (Y)	48 ±3.4	47 ±2.1
Fracture lower limb	Left / right	14 (61%) / 9 (39%)	44 (53%) / 39 (47%)
	Mid tibia	6 (26%)	32 (39%)
	Distal tibia & malleoli	8 (35%)	30 (36%)
Cause of injury	MBA	8 (35%)	25 (30%)
	MVA	5 (22%)	19 (23%)
	Crush	5 (22%)	10 (12%)
	Other (fall)	5 (22%)	29 (35%)
Adverse event	Soft tissue infection	9 (39%)	22 (79%)
	Partial necrosis	6 (26%)	8 (30%)
	Osteomyelitis	8 (35%)	11 (41%)
	Infected hardware	3 (13%)	14 (52%)
Outcome upon discharge	Bone union 3/12	6 (26%)	23 (36%)
	Delayed union	5 (22%)	23 (36%)
	Infected non-union	3 (13%)	12 (19%)
	Non- weight bearing	8 (35%)	32 (42%)
	Partial weight bearing	7 (30%)	34 (44%)
	Non weight bearing	3 (13%)	32 (42%)
Mean time	Length of hospital stay (D)	29 ±4.5	28 ±3.0
	Accident -> theatre (H)	8 ±1.1	9 ±0.6
	Admission -> theatre (H)	4 ±0.7	5 ±0.4
	Internal fixation (D)	5 ±1.8	4 ±0.9
	Soft tissue coverage (D)	7 ±1.2	8 ±1.0
Internal fixation	Nail	4 (17%)	36 (47%)
	Plate	8 (35%)	29 (38%)
	Other	6 (26%)	24 (32%)
Soft tissue reconstruction	Mean VAC (D)	7 ±1.2	7 ±1.0
	Free muscle flap	14 (61%)	58 (48%)
	Local reconstruction	9 (39%)	37 (31%)

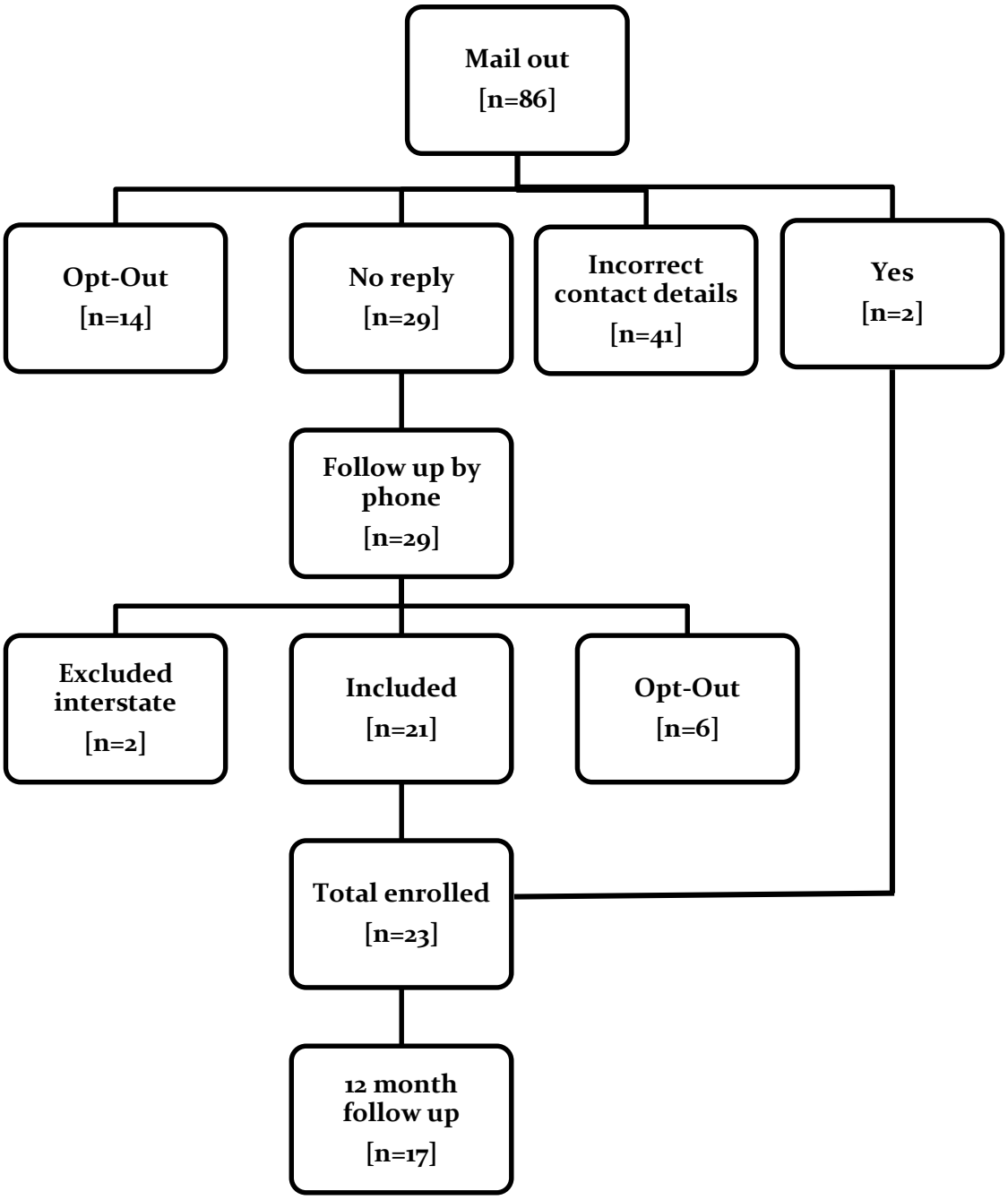

Figure 5-1 Recruitment flow chart.

The study participants mirror the recruitment database with more males than females (83% versus 17%), a mean age of 44 years and left leg fracture being more common than right (61% versus 39%). The most common trauma in the study participants was MBA (35%) followed closely by MVA (22%) and a range of work related crush injuries (22%) (Table 1)

Soft tissue infection was evident in 79% in the recruitment database and 39% in the study participants. The high percentage of infection in the recruitment database can be explained by the fact that 55 patients had no information regarding soft tissue infection and subsequently this was counted as "missing" in the database. This resulted in a final total of 28 of which 79% (N=22) was "yes" for soft tissue infection.

Osteomyelitis was reported in 41% of the recruitment database and in 35% of the study participants. At the first follow up, 36% (n=23) had bone union. 36% (n=23) of patients in the recruitment database had delayed bone union and 19% were identified as infected non-union. Upon discharge it was noted that 44% (n=34) were able to partially weight bear but 42% (n=32) were non-weight bearing.

In the recruitment database the overall mean (SE ±) length of hospital stay was 28 (±3.0) days (range 3 -170 days). The most common bone fixation was found to be nail (47%, n=36) followed by plate (38%, n=29) and (32%, n=24) other, such as Kirschner wire or K-wire.

In the study participants group the overall mean (±SE) length of hospital stay was 29 (±4.5) days (range 7 - 97). Plate and screws (35%) for internal fixation were more often utilised rather than nail (17%). This may be due to the complexity of the fractures specifically in this study group. As it can be seen from the location and bones involved in the fracture; tibia and fibula are most evident in the study group (43%).

Mean (±SE) days of Vacuum Assisted Wound closure device was found to be 7 days (±1.0) with a range of 0 to 53 days. Most patients received a free muscle flap reconstruction (48%,

n=58) and (31%, n=37) a local soft tissue reconstruction. This was consistent with the study participants group who had 61% free muscle flaps and 39% local flaps.

5.3 Results: IMP™ SFB7 Bioimpedance Spectroscopy (ImpediMed Ltd, Queensland, Australia).

Bioimpedance Spectroscopy (BIS) measurements were undertaken to measure extracellular fluid (ECF) in the lower extremities and record difference between the affected (reconstructed) leg with the contralateral leg acting as control. Electrodes were placed according to SFB7 instructions (Chapter 4.2.2), using wrist to ankle fixated electrodes with the participant in a supine position. Baseline measurements were taken, but logistical problems such as interference by metal implants, meant only 16 out of 23 participants had acceptable BIS measurements at baseline; an additional 4 datasets were not acceptable at follow up. General participant details such as gender, age, mean weight and height and mean BMI at baseline and at the 12 month follow up are summarised in Table 5-2.

Table 5-2 : Summary of participants characteristics presented as mean, % percentage and ± Standard Error at baseline and follow up.

Characteristics	Baseline (male / female)	Follow up (male only)
Gender (male / female)	13 (82%) / 3 (18%)	13 (100%)
Mean age (y)	44 ±4.7 / 54 ±9.2	54 ±3.7
Mean weight (kg)	87.4 ±4.4 / 80.6 ±1.3	91.2 ±4.2
Mean height (cm)	175.4 ±2.0 / 159.7 ±4.4	175.2 ±1.9
Mean BMI	28.4 ±1.4 / 31.8 ±1.8	29.6 ±1.1

The affected leg shows a significantly lower impedance value (R_0 in units ohm) compared to the control leg at baseline and follow up (Table 5-3).

A “low resistance” value indicated a higher conductivity and thus a larger presence of extracellular fluid in the affected limb. The difference between the affected leg versus control at baseline and follow up was statistically significant (P<0.005 and P<0.05).

A further BIS measure of the area below the knee was undertaken at follow up to identify if there was any increased extracellular fluid within this area (Table 5-4). Due to reduced number of participants in the follow up the mean age shifted from 44 years at baseline to 54 years at follow up. The mean extracellular fluid was higher below the knee in the affected leg and this was statistically significant.

Table 5-3 Bioimpedance spectroscopy R0 extracellular fluid affected leg versus control at baseline (n=16) and follow up (n=13). Paired T-test.

Bioimpedance (extracellular fluid)	Mean (Ohm) ±SE Baseline (n=16)	Mean (Ohm) ±SE Follow up (n=13)
R0Affected leg	227.4 ±14.2	169.3 ±9.6
R0Control	275.1 ±11.8	211.7 ±14.2
Mean difference	48.1 ±8.2	33.1 ±10.0
P-value	<0.005	<0.05

Table 5-4 Bioimpedance spectroscopy R0 extracellular fluid below the knee affected leg versus control at follow up (n=10). Paired T-Test.

Bioimpedance (extracellular fluid below knee)	Mean (Ohm) ±SE Follow up
R0Affected below the knee	115.5 ±11.0
R0 Control below the knee	144.5 ±10.5
Mean difference	29.0 ±11.5
P-value	<0.05

5.4 Results: Moisture Meter D® (Delfin Technology Limited, Kuopio, Finland)

At both the baseline and follow up measurement, the tissue di-electric constant (TDC) values and local tissue water (LTW) percentage in the affected leg were significantly higher than the

non-affected leg (P<0.005). Using a linear mixed effect model adjusted for body mass index (BMI) the difference between the affected and non-affected leg remained significantly different (P<0.005) at baseline and follow up (Table 5-5). Adjusting for BMI did not change any of the coefficients.

Table 5-5: Moisture meter; Tissue Di-electric Constant and Local Tissue Water measured at baseline (n=19) and follow up (n=17). TDC data and standard error between control and affected leg adjusted for BMI with ±SE. Linear mixed effect model.

Tissue Di-electric Constant (Local Tissue Water)	Baseline mean ±SE (LTW% ±SE)	Follow up mean ±SE (LTW% ±SE)
Affected leg	41.7 ±1.4 (52.6% ±1.8)	41.3 ±1.4 (52.0% ±1.8)
Control leg	27.7 ±1.4 (34.4% ±1.8)	30.1 ±1.4 (38.0% ±1.8)
Mean difference	14.1 ±1.1 (18.1 ±1.4)	12.4 ±0.7 (14.5 ±1.2)
P-value	<0.005	<0.005

The mean LTW percentage and standard error of the affected leg versus the control leg from baseline to follow up is indicated in brackets in Table 5-5. As expected, these findings are similar to the TDC values as it is merely a different unit of measurement of the same core parameter, but a unit more representative of the true difference in local tissue water between the affected area and control (1).

5.5 Results: Vapometer® (Delfin Technology Limited, Kuipio, Finland)

Measuring trans epidermal water loss (TEWL) on the reconstructed site and control leg presented some difficulties. These include hair growth, bulky scar tissue, scaly, dry or recently moisturised skin all of which resulted in a large fluctuation of the TEWL readings. The outcome results of 3 repeated measurements at follow up on the control leg and affected leg are indicated in Figure 5-2.

The coefficient of variation for the Vapometer measurements ranged up to 1.9. Therefore, further analysis was not performed as the repeatability of Vapometer data proved to be poor.

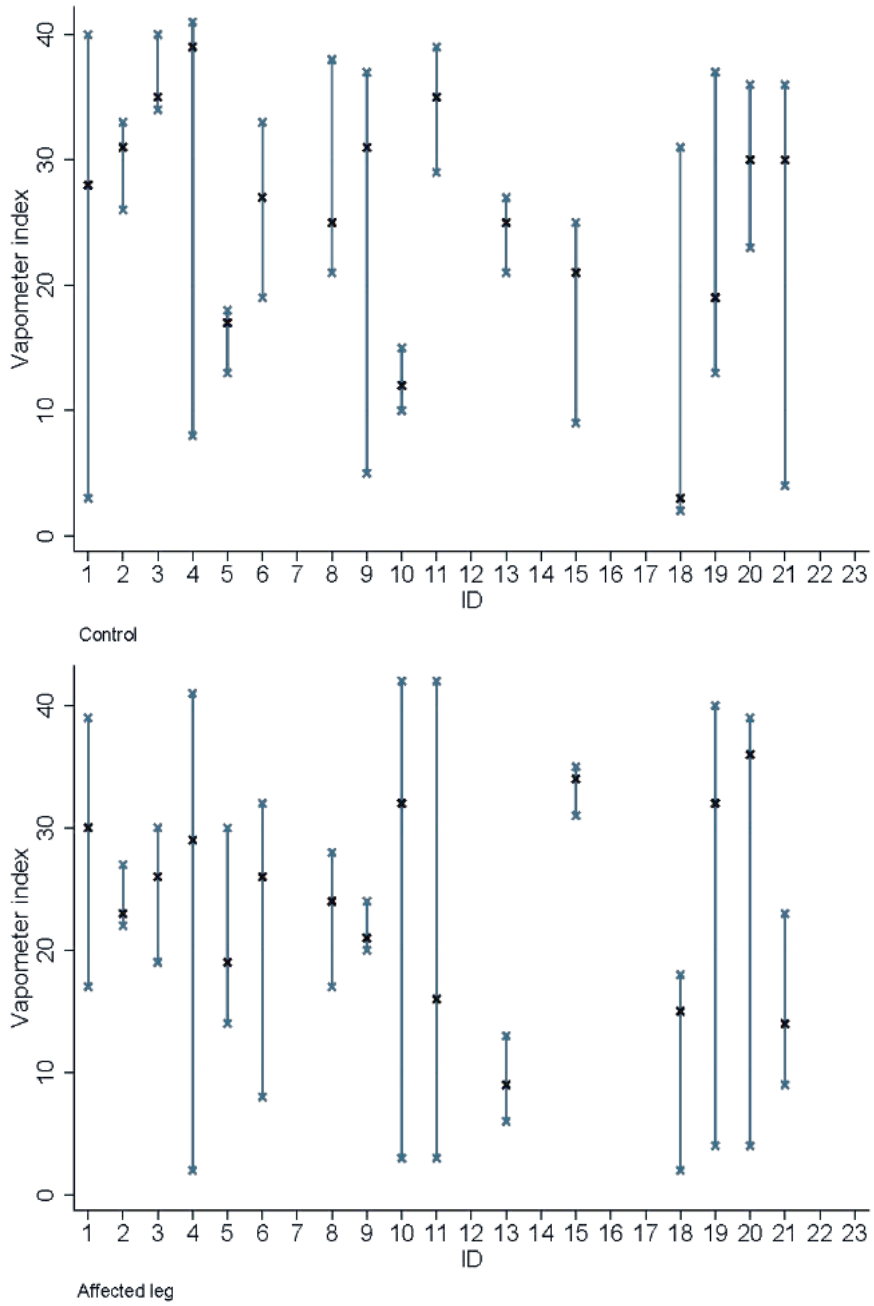

Figure 5-2: Baseline scatterplot with 3 repeated measurements by participant ID. Vapometer units represented as g/m²/h[1].

5.6 Results: Circumferential measurements

The circumference of both legs was measured every 4cm with a BSN Medical JOBST® generic measurement tape starting from 10cm from the heel up to the maximum length of the leg. A truncated cone formula was applied to the circumferential data to calculate the volume of the leg. Participants 6, 11, 16, 21 and 23 were lost to, or opted out, of follow up and therefore

have no follow up data. Participant 13 had an amputated control leg and was therefore not included in the circumference measurement. The general observation from this data was that the majority had larger volume in their affected leg compared to the control leg. At baseline, two participants (16 and 11) had a larger volume in the control leg than the affected leg; therefore, a negative value is depicted (Figure 5-3). In one participant (11) the whole control leg (measured from ankle to hip) volume was greater than the affected leg, but there was a higher volume in the affected leg below the knee compared to the control leg. Participant 1 showed a higher volume in her affected leg below the knee at baseline, however at follow up it seemed that both the whole leg and below the knee of the control leg indicated a higher volume than the affected leg. In participant number 7 at baseline, the affected whole leg contained over 692ml more volume than the control leg, and the below the knee volume was also greater (379ml difference, Figure 5-3). At the 12 month follow up, this participant had gained 6 kilos' and also started a new job which involved more standing resulting in both legs having more volume, but the control leg having 1861 mL more volume than the affected leg.

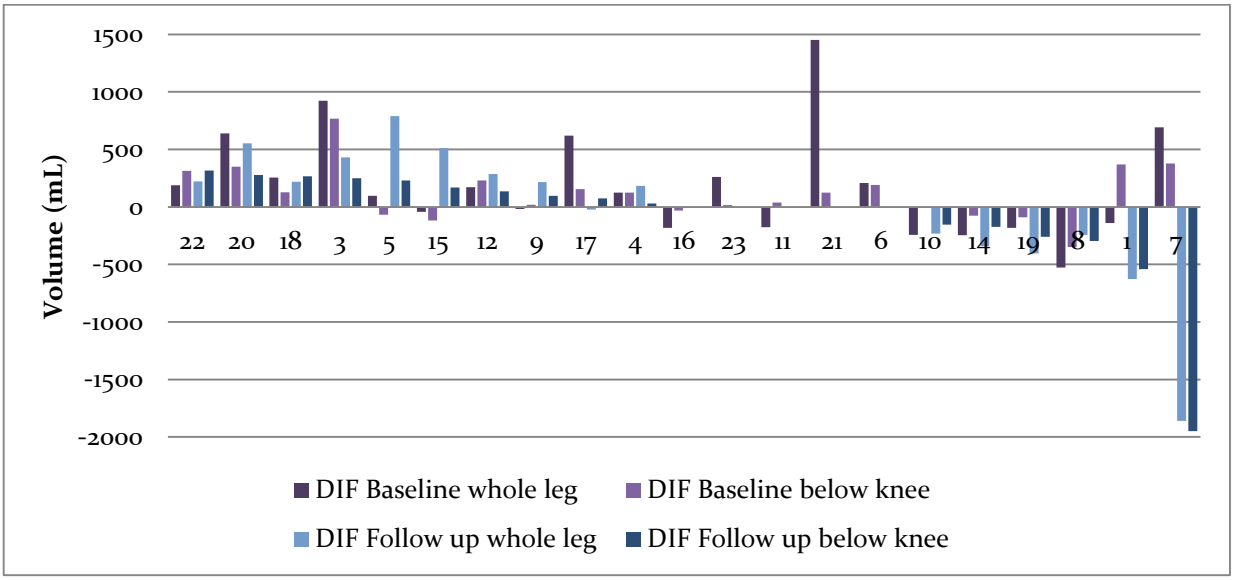

Figure 5-3: Volume difference (DIF) in mL between affected leg and control. Whole leg at baseline and follow up and below the knee volume at baseline and follow up. Data is for individuals specified by ID number.

5.7 Results: Indocyanine Green lymphatic imaging

Out of the total 23 participants that were able to attend and complete the study assessment 19 were eligible for Indocyanine Green (ICG) lymphatic imaging. Four participants were excluded from ICG imaging, due to flap location on the heel, active hepatitis B infection, neuralgia around the reconstructed site or a history of recurrent cellulitis. Of the remaining included participants that had reconstructive surgeries (Table 5-6); 10 participants received a free muscle flap reconstruction with split thickness skin graft, 1 free fasciocutaneous flap, 8 local reconstructions. The local reconstructions cover; 1 full thickness skin graft, 6 local fasciocutaneous flaps and 1 local gastrocnemius rotation flap.

The majority of ICG imaged patients were male (n=17, 90%). The mean age at presentation was 48 year (range 26-73 years). The mean follow-up time between accident and first study assessment was 32 months (range 2-62 months). Vacuum Assisted Closure (VAC) prior to the reconstruction was used for an average of 5 days (range 2-50).

All participants showed functional differences between the affected and non-affected leg ICG images. On average, ICG fluorescence was observed to be slower in crossing from the injection site over the talus in the affected leg compared to this same pathway in the normal contralateral limb, suggesting a reduction in the transport capacity. Of the participants with free muscle flap reconstruction, 6 showed a dermal backflow pattern in the peri-reconstructed area (Figure 5-4, 5-5, 5-6). The remaining 4 free muscle flap participants showed lymphatic vessels bypassing the reconstructed site. Interestingly no functional lymphatic vessels were noted within any of the free muscle flaps.

*Table 5-6: Reconstruction type by patient ID, gender (Gen), age, mechanism of injury (Mech); motor vehicle accident (MVA), motorbike accident (MBA),follow up time from reconstructive surgery to baseline study assessment, internal bone fixation – intramedullary nailing (IMN) * external fixation, type of reconstruction infection, and time to final flap coverage in days and flap size if known (width by height in centimetres).*

ID	Gen	Age	Mech	Follow up	Fixation	Recon	Infection	Days	Size
1	F	70	MVA	36	Plate / IMN*	Local Gastroc	No	4	5.5X6.5
2	M	73	MVA	44	Plate*	Ant Lat Thigh	No	11	16X13
4	M	34	Metal Cable	39	IMN	Local flap	Osteomyelitis	1	
5	M	68	Fall	39	IMN	Local flap	Soft tissue	3	14
6	M	33	Crush	39	Plate	Local flap	No	4	
7	M	26	MBA	30	Plate*	Gracilis	Osteomyelitis	4	11.5X14.5
8	F	38	MBA	31	IMN	FT graft	No	1	5.2X12.5
9	M	33	MBA	31	Plate*	Local flap	No	8	
10	M	44	Fall	52	IMN	Local flap	Osteomyelitis	6	9.5X7
12	M	65	Crush	29	*	Local flap	No	5	6.5X10
13	M	28	MVA	62	Plate	Lat Dorsi	Osteomyelitis	18	25.5X12
14	M	71	Fall	10	Plate	Gracilis	Soft tissue	15	
15	M	59	MBA	44	IMN-Plate*	Lat Dorsi	Osteomyelitis	8	19.5X17
16	M	55	MVA	14	Plate	Gracilis	Soft tissue	19	13X14
18	M	49	Fall	41	Plate	Gracilis	Osteomyelitis	9	16X20
19	M	56	MVA	36	Plate	Lat Dorsi + RFF	Full thickness necrosis	7	13.5X16.5 7.5X20
20	M	37	Crush	26	Plate*	Lat Dorsi	Osteomyelitis + soft tissue	22	17.5X19.5
22	M	28	MBA	2	IMN*	Lat Dorsi	No	8	9X24
23	M	49	MBA	2	IMN	Gracilis	Osteomyelitis	-	

All local flaps, including the full thickness skin graft of one participant, had scar tissue that was largely devoid of visible superficial lymphatic vessels. The lymphatic vessels that were present appeared torturous, bypassing the scar tissue and the reconstructed area completely. In 2 cases lymph flow was diverted from the great saphenous vein area towards the posterior side of the leg to follow the small saphenous vein. This pattern of drainage might be expected when injection occurs near the Achilles tendon, however in these cases the injection was on the dorsum foot making the observed drainage pattern unusual. 1 case demonstrated retrograde flow towards the sole of the foot as the scar tissue and flap covered most of the ankle (Figure 5-6).

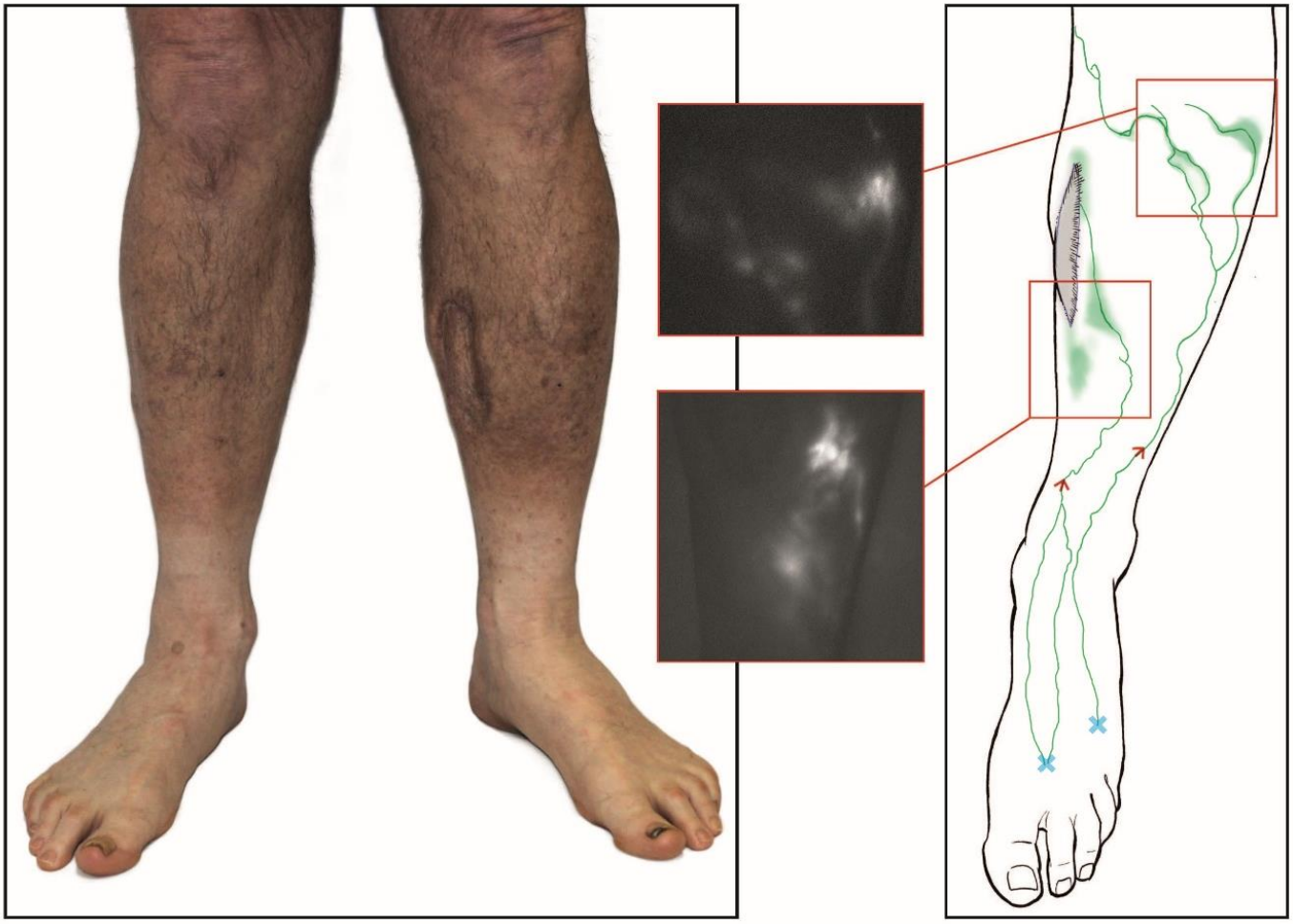

Figure 5-4: Local reconstruction (ID 12): From the point of injection (blue X) on the dorsal foot proximal to the toes, uptake of ICG followed the anatomical lymphatic drainage pathway (green lines with red arrows representing direction). Patches of dermal backflow are observed (red outlined boxes) distally from the knee and dermal backflow around the flap. No superficial lymphatic activity observed within the reconstructed site (6).

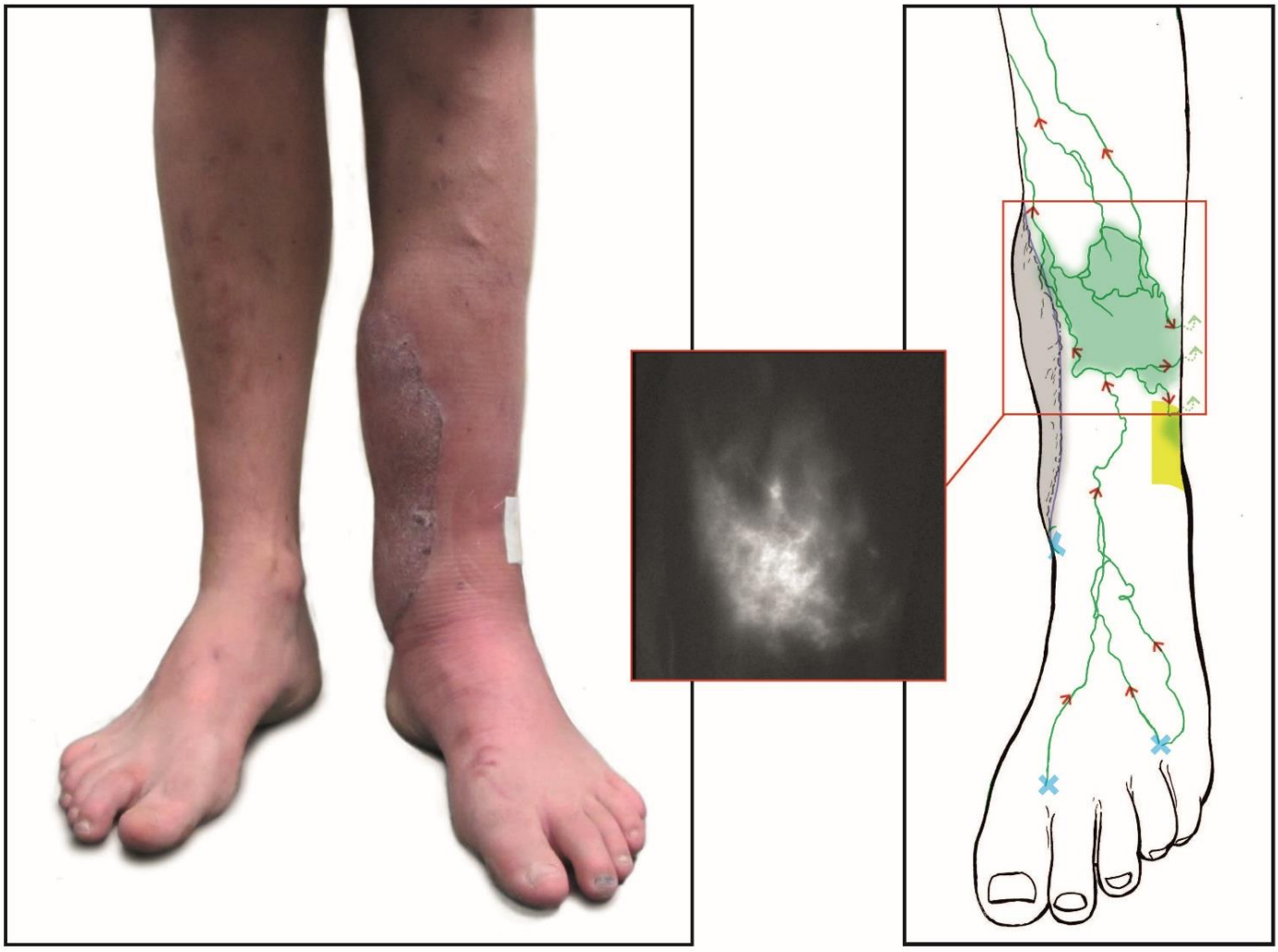

Figure 5-5: Free muscle flap (ID 22): From the dorsal foot injection site and the additional distal flap injection (blue X) the ICG uptake reaches mid tibia where the proximal flow ceases and shows, pooling of lymph in a stardust like pattern (red outlined box lateral to the muscle flap). From the stardust pattern, two vessels emerge and continue following the great saphenous vein anatomy converging with a lymphatic vessel that seems to have followed the lateral side of the muscle flap (6).

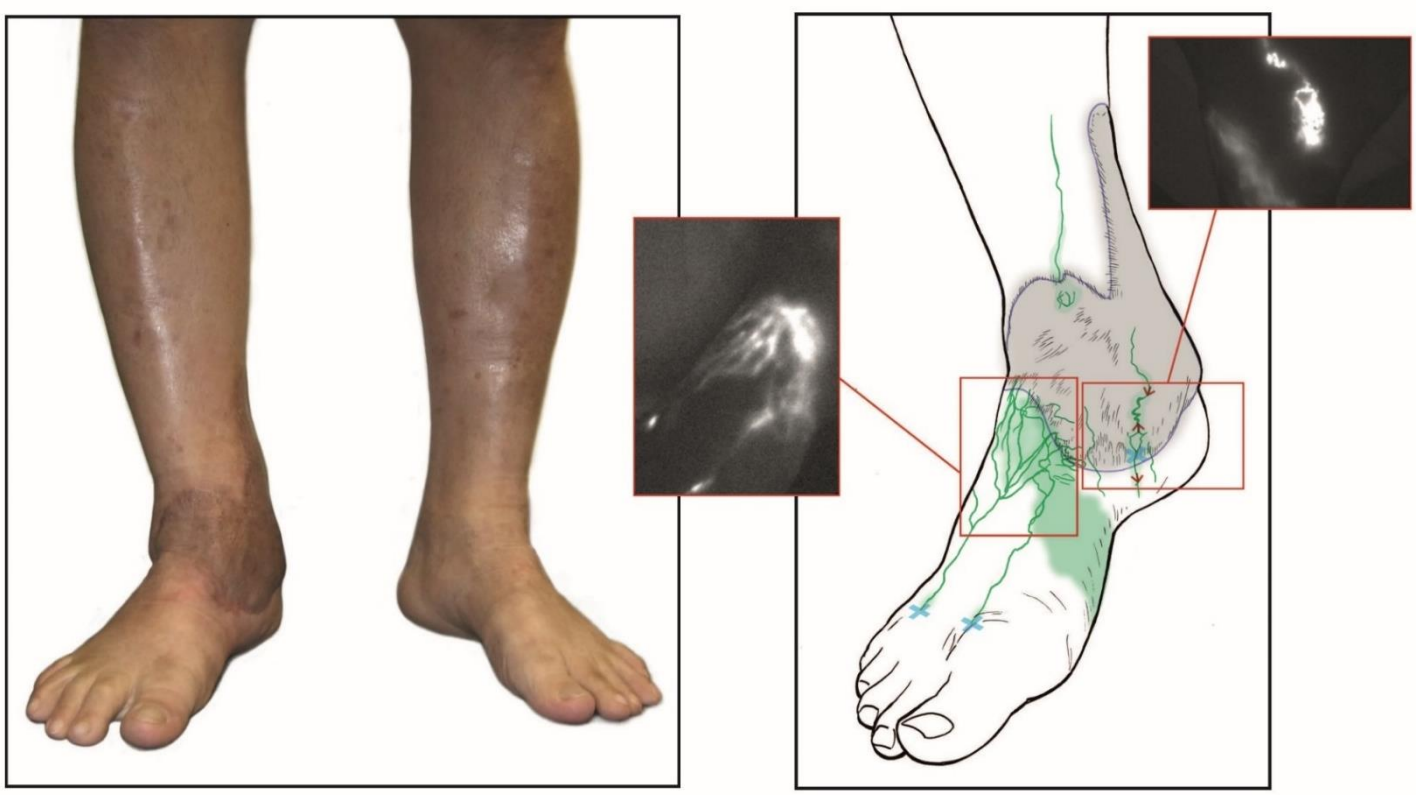

Figure 5-6: Free muscle flap (ID 15): From the point of injection on the dorsal foot proximal toes and distal flap (blue X) shows blockage of the superficial flow and dermal backflow patterns. From the distal flap injection site, one lymphatic vessel seems to be active within the flap but the flow returns distally after it reaches halfway into the muscle flap. It continues towards the sole of the foot (6).

At the 12 month follow up there was no observable change from the baseline lymphatic pattern.

Participant 22 (Figure 5-7 a) who was initially imaged 2 months post his reconstructive surgery showed clinically visible swelling at baseline evidently caused by the surgery. The 12 month follow up showed the same patterns of dermal backflow but smaller (Figure 5-7 b). At follow up the participant was weight bearing and returned to work with reduced hours.

5.7.1 Additional lymphoscintigram data

At the 12 month follow up, 3 participants (2, 17, 18) were identified as having clinically visible persistent swelling in the reconstructed leg consistent with lymphoedema. The ICG image in 2 participants (1 excluded for the ICG imaging due to dark skin and active Hepatitis B) showed dermal backflow pattern in and around the reconstructed area. The participants were referred back to the treating specialist and were booked in for lymphoscintigram at the Royal Adelaide

Hospital Nuclear Medicine Department. Images were obtained with Lymph-Flo (Technetium [^{99m} Tc] Colloidal Antimony Sulphide) injection with a near ideal particle size of approximately 10nm (2). Intradermal injections were administered into the first web space of both feet and migration of the radio colloid was traced with a gamma camera at 30 minutes, 120 minutes and in 1 case at 240 minutes. Participants were encouraged to walk as much as possible between imaging.

The three participants had lymphoedema confirmed by the lymphoscintigram results. Participant 2 showed dermal backflow patterns consistent with his ICG image of dermal backflow and constriction of the large lymphatic collector around the popliteal area of the affected leg. Migration of the tracer into the lymph nodes in the iliac and inguinal region was slower in the affected limb compared to the contralateral control limb. The structure of the lymphatic system from the knee up appeared normal. Participant 17 showed dermal backflow below the knee, visualised as a "hotspot" of the tracer proximal of the flap reconstruction as well as 3 active popliteal lymph nodes in the affected leg. Overall the lymphatic collectors showed a slight abnormality even above the reconstructed area. Participant 18 (Figure 5-8, 5-9) experienced discomfort from clinically visible swelling mostly in the reconstructed area but also in the whole region below the knee. The lymphoscintigram showed dermal backflow below the knee, a slow migration of the tracer in the affected leg with limited uptake in the iliac and inguinal lymph nodes at 120 minutes' post injection (Figure 5-10).

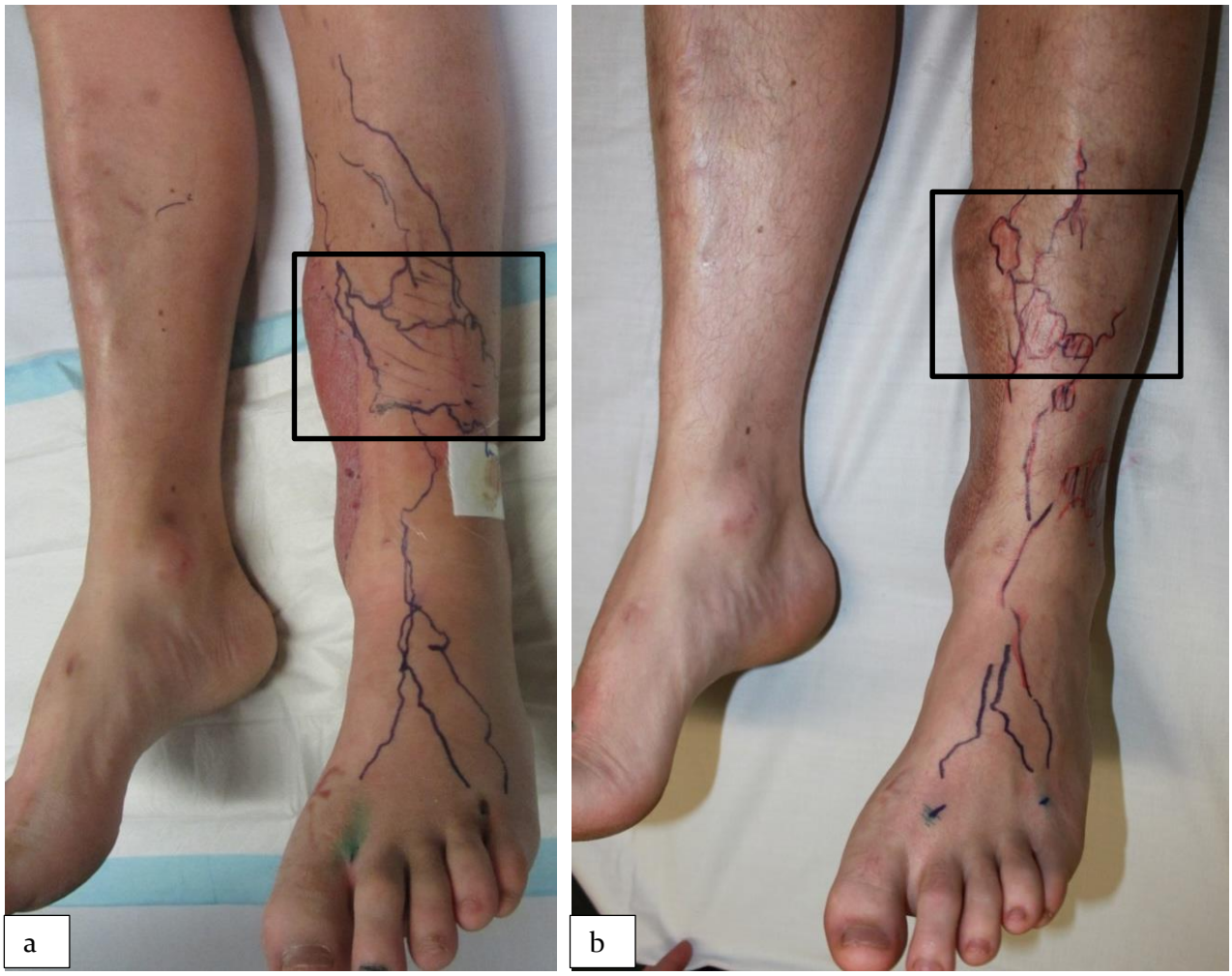

Figure 5-7: Free muscle flap (ID 22). 7A lymphatic imaging pattern at baseline injection near the base of the toes (first and fourth web space) black square represents area of dermal backflow. 7B lymphatic imaging pattern at 12 months follow up with same injection location. Black square indicates area of dermal backflow which is similar area as 12 months prior but closer to the flap edge.

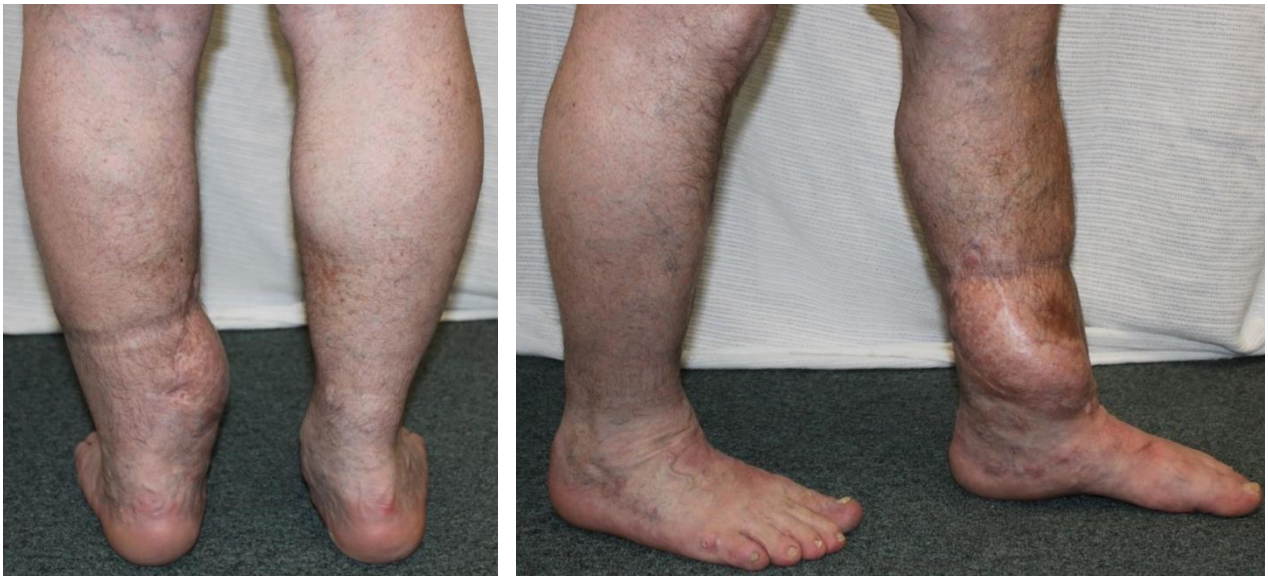

Figure 5-8: Free muscle flap (ID 18). Clinically visible swelling in and around the reconstructed region.

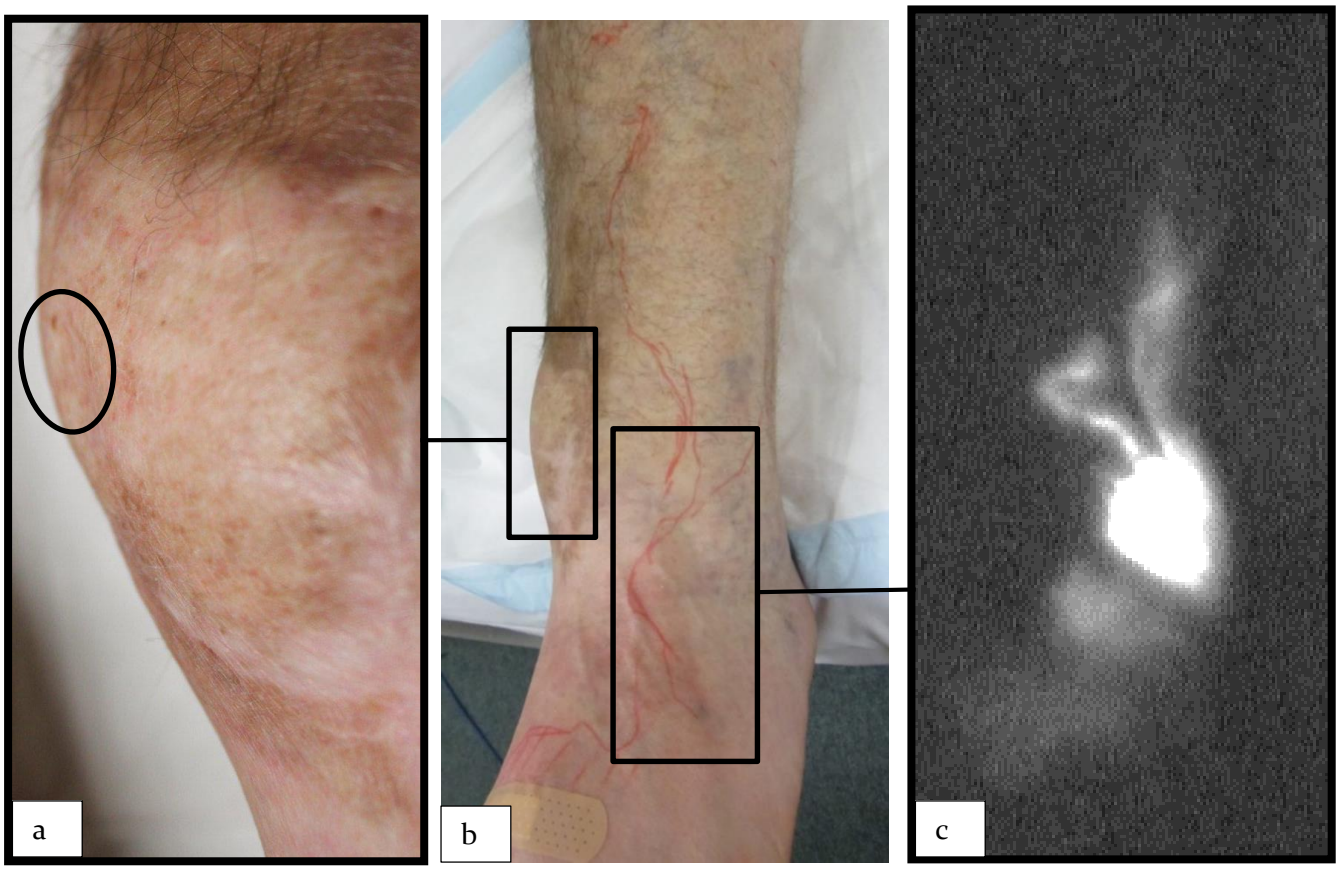

Figure 5-9: Free muscle flap (ID 18). 9A: Pitting oedema in the flap. 9B location of the flap and drawn ICG image in red. 9C ICG pattern from injection; deviating from the reconstructive area.

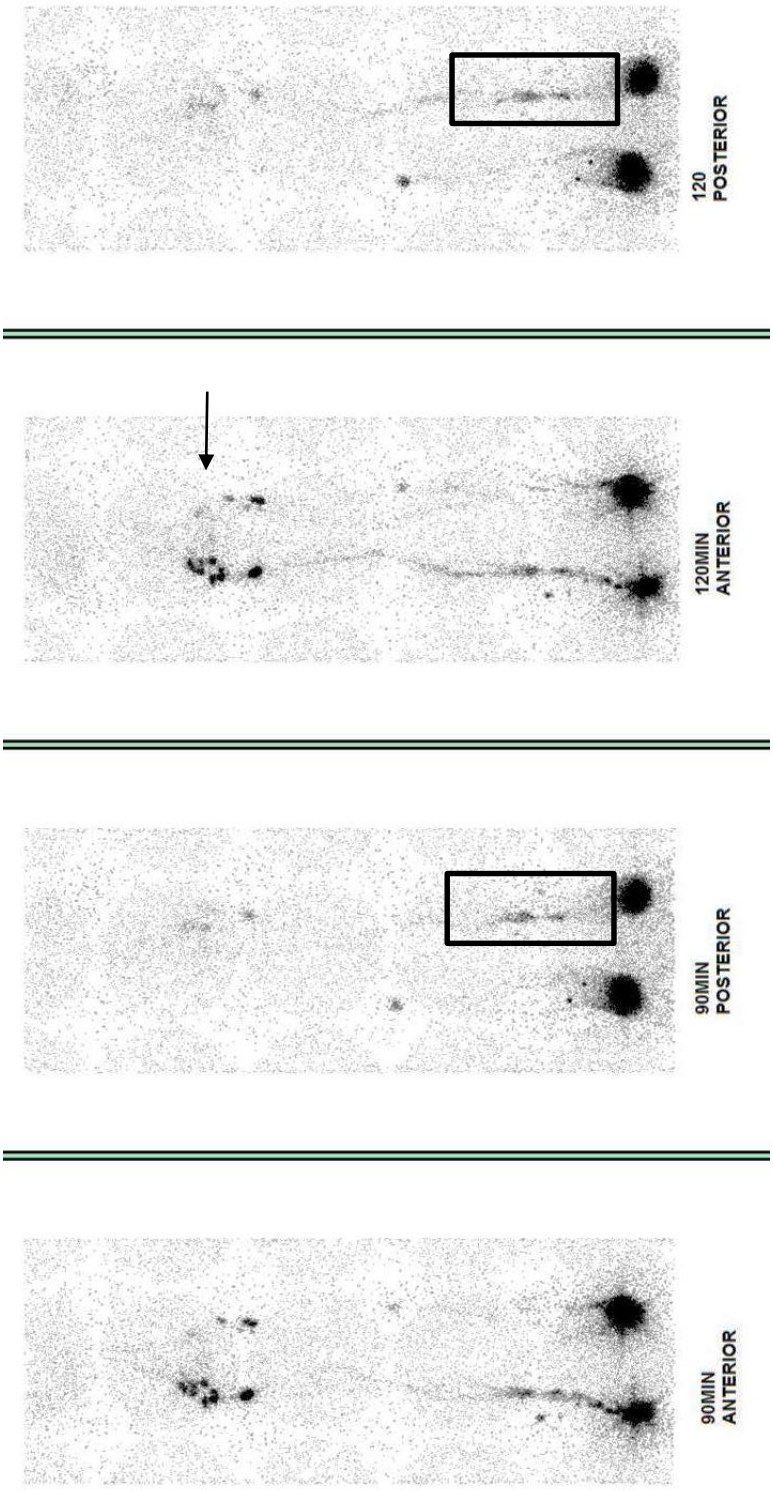

Figure 5-10: Lymphoscintigram details (ID 18). Clinically visible oedema confirmed by lymphoscintigram with obvious dermal backflow at distal end of affected leg around the reconstructed area (box). Also there is a limited uptake of the tracer into the lymph nodes on the affected side (arrow) after 120 minutes.

5.8 Results: Qualitative data

Two self-administered questionnaires were completed at the time of baseline assessment and at follow-up. These were a 12 question general quality of life or Short Health Form (SF-12) and the 20 question Lower Extremity Functioning Scale (LEFS).

At baseline, all participants were asked a series of questions as part of a general medical history. A trend of functional problems over the 23 participants was seen in the questions regarding pain, and details of swelling. 78% (n=18) of participants reported having pain in the leg related to the trauma, 9% (n=2) reported no pain and 13% (n=3) reported discomfort or cramps in the affected leg. Prior to the clinical assessment, all participants were asked if they had experienced any swelling in the reconstructed area or leg; 96% (n=22) answered with "yes" and one "no".

With respect to the location of the swelling and its severity participants could select more than one answer. 74% (n=17) answered the swelling is "mostly in reconstructed area", 52% (n=12) mentioned the swelling was "mostly below the knee", and 9% (n=2) reporting to have swelling in the whole leg. The majority reported the "swelling is worse in the evening but resolves in the morning" (70%, n=16) and nearly half experienced "more swelling when it is hot" (48%, n=11).

5.8.1 Results: short general health questionnaire (SF-12)

From the data set of 23 patients (1 missing due to time restrictions) each received a SF12 questionnaire that was self-administered. Patients were re-assessed after 12 months with a response rate of 77% (n=17).

Data was scored with Health Outcome Scoring software available from Quality Metrics. Results of the initial assessment (n=22) was a mean Physical Component Summary (PCS) of

39.19 (±SE 1.8) with a range of 22.13 to 56.58. The mean Mental Component Summary (MCS) was 48.30 (±SE 2.8) with a range between 22.61 and 67.13.

At the 12 month follow up (N=17) the mean PCS score was 42.09 (±SE 2.4) with MCS score 53.72 (±SE 1.77). No statistical significance was found between PCS baseline and PCS follow up (Paired T-Test, P= 0.5894) or MCS baseline and MCS follow up (Paired T-Test, P= 0.2876). These mean results are comparable with the known healthy South Australian population SF-12 data which reported a mean PCS of 48.9 and mean MCS of 52.4 respectively (3, 4).

5.8.2 Results: Lower Extremity Functioning Scale (LEFS)

The LEFS is a validated measurement tool to score activity limitation in participants who have had lower extremity trauma. The score per question is from 0 ("unable to perform" or "extreme difficulty) to 4 ("no difficulty") and the total score can be up to 80 with 80 representing low level of activity limitation. From the dataset of 23 there was a 100% completion rate with the mean score of all patients at baseline being 42 (out of 80). The median score at the 12 month follow up was of 58 out of 80 (Table 5-7).

Table 5-7 The mean score (±SE) and percentage of maximum functioning of the lower extremity functioning scale (LEFS) at baseline (n=23) and follow-up (n=17). Self-administered questionnaire.

LEFS	Mean score (± SE)	Max functioning (%)
Baseline total (n=23)	45.3 ±3.8	56.6%
Follow up total (n=17)	53.8 ±4.5	67.2%
Mean difference	4.2 ±2.4	24.7%
P-value	0.1044	-

At baseline 10 participants scored 40 or lower; with 6 between 40 and 60, and 7 with 60 or higher. At follow up 6 scored 40 or lower, 3 between 40 and 60 and 7 scored 60 or higher. Specifically most of the low scores ("unable to perform", "extreme difficulty") were identified

in the last few questions concerning the following items: “running on even ground”, “running on uneven ground”, “making sharp turns while running fast” , and “hopping”.

The Paired T-Test did not show any statistically significant change between the baseline and follow up (P= 0.1044). A few of the participants showed a greater than 9 points difference on the LEFS which represent a clinically detectable change (5). Participant 18 had worsening clinical symptoms of the swelling in the affected leg at follow up compared to baseline. However, the follow up result of the LEFS showed *improvement* of his functioning. It was noted that the last 5 questions were scored with 0 at baseline (“unable to perform” or “extreme difficulty”) and at follow up scored with 4 (“no difficulty”). There is a likely chance of the participant misunderstood the question/misinterpreted the answers. Participant 12 showed LEFS improvement with 14 points and participant 22 was assessed at baseline only 2 months post-surgery therefore an improvement of 18 point in the LEFS is expected at the 12 month follow up (Figure 5-11).

5.9 Discussion

The clinical study showed that severe lower leg trauma has a significant impact on an individual’s appearance and function, as well as affecting extracellular fluid and lymphatic vessel function. There were measurable similarities and trends within the lower leg trauma group, including the lymphatic vessel structure and patterns seen with indocyanine green (ICG) lymphatic imaging, high extracellular fluid measured with bio-impedance and increased local tissue water within the reconstructed site measured with the moisture meter. However, there were also large variation within this pattern. These are important to investigate, on a case by case basis to highlight the individuality of each specific case in this cohort.

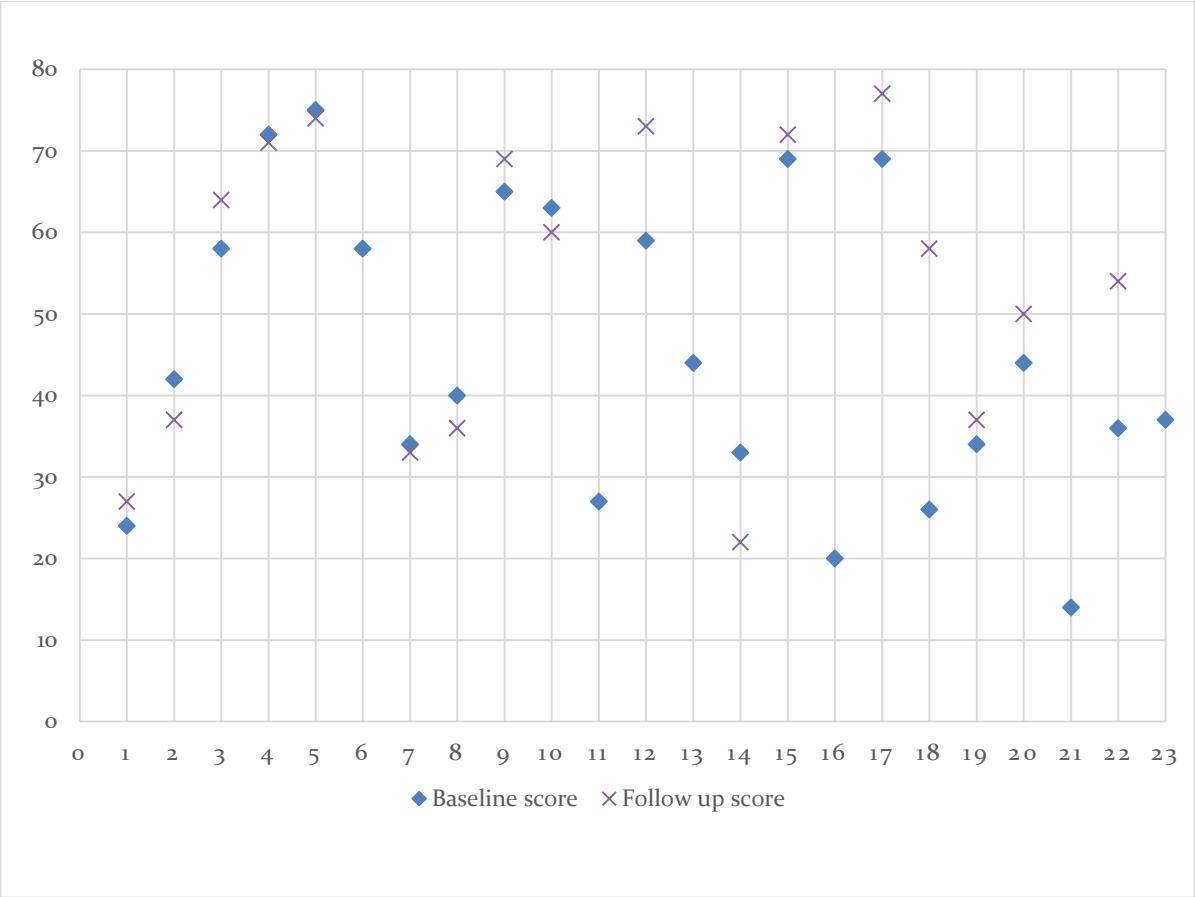

Figure 5-11 Individual scores of LEFS at baseline (blue diamond) and at follow up (purple cross).

The self-reported pain and swelling at the initial general medical history interview showed that nearly all participant *experienced* swelling. Not all participants had visible swelling at clinical assessment, but perhaps the 'feeling' of the reconstructed site may come across as swelling whereas it also might be the bulkiness of the flap itself and/or the altered nerve sensation of the reconstructed area. Most participants also mentioned having severe swelling after the surgical intervention(s) for as long as 18 months which rapidly (in some) reduced when they were able to walk again

Additional to those in the study group there have been phone conversations with patients who since the accident moved interstate and were therefore excluded. In conversations with these patients, the general themes were a "sensation" of swelling of the flap area (often referred to as 'lump" on the leg) or the leg swelling in hot weather or at the end of the day.

Some of these patients were wearing a compression garment provided by their physiotherapist or one or two compression bandages (low compression tubular bandage) that were provided at time of discharge at the hospital.

Missing Moisture Meter data was related to another trial using the equipment (n=6) or in case of the Bio Impedance, data were incomplete when large surface of metalwork such as metal tibial plates interfered with the Cole-Cole plot reading (n=7). The low follow-up percentage was due a range of reasons, a patient in legal action that was advised not to be measured again. A patient with post-traumatic stress reliant on a wheelchair did not have the energy to come in for the research again. Other participants opted in for the follow up measurement under the condition not to get the ICG imaging again due to discomfort they experienced with the injection (n=5). This could be explained as some participants had ongoing pain in general and/or hypersensitivity distal from the reconstructed site. And finally one participant was measured just before the closure of the study therefore a 12 month follow up was not possible.

Although the physical and mental component summaries of the SF-12 did not show much deviation from the healthy South Australian population it was clear in the clinical study that some of the participants struggled to return to work, were trying to cope with pain and/or reduced mobility. This was also observed in the recruitment phase where participants were followed up by phone. Some verbally opted out based on being mentally traumatised by the accident or having undergone recurrent reconstructions of either the joints affected by the accident (such as hip or knee reconstruction) or soft tissue reconstruction itself.

5.10 Chapter conclusions

This research has specifically targeted patients with extensive soft tissue loss and reconstructive surgery.

It is clearly demonstrated in this clinical study that lymphatic flow is affected by reconstructive surgery following deep soft tissue injury. It is also clear that scar tissue is obstructive to normal lymphatic flow and that free muscle flaps seem to have no normally functioning lymphatic vessels. Fluid specific measurements show statistically significantly more fluid in the affected limb compared to the contralateral control limb. The lower extremity functioning scale showed that participants were at 56.6% of the maximum functioning at baseline with 67.2% of maximum functioning at the 12 month follow up. Differences between affected and control leg volume was observed with the majority having a larger volume in the affected leg.

References

1. Mayrovitz H, Weingrad D, Brlit F, Lopez L, Desfor R. Tissue dielectric constant (TDC) as an index of localized arm skin water: differences between measuring probes and genders. Lymphology. 2015;48(1):15-23.

2. Tsopelas C, Smyth D. Lymph-Flo 2016 [Available from: https://www.rah.sa.gov.au/nucmed/radpharm/rph_products.htm.

3. Sanderson K, Andrews G. The SF-12 in the Australian population: cross-validation of item selection. Australian and New Zealand journal of public health. 2002;26(4):343-5.

4. Avery J, Del Grande EA, Taylor A. Quality of life in South Australia as measured by the SF-12 Health Status Questionnaire : population norms for 2003 : trends from 1997-2003. South Australia: Population Research and Outcome Studies Unit; 2004.

5. Binkley JM, Lott SA, Riddle DL, Stratford PW. The Lower Extremity Functional Scale (LEFS): Scale Development, Measurement Properties, and Clinical Application. Physical therapy. 1999 1999/04//:371.

6. Van Zanten M, Mistry R, Suami H, Campbell-Lloyd A, Finkemeyer J, Piller N, Caplash Y. "The lymphatic response to injury with soft tissue reconstruction in high-energy open tibial fractures of the lower extremity". Journal of Plastic and Reconstructive Surgery (PRS). *In press.*

CHAPTER 6
Discussion and conclusion

This Chapter summarises this research and discusses in detail the contribution of the previous chapters as well as the discussion of the study limitations, and future directions for research into the lymphatic system and its response to trauma.

6.1 Summary

This study explored the influence of trauma related lower limb reconstruction on the lymphatic system. A variety of measurement tools have been used to investigate the presence of local and general oedema, to objectively investigate the individuals' general health and lower limb functioning after trauma. This research has contributed to a significantly improved understanding of the extent of damage to the lymphatics through soft tissue loss and in the subsequent reconstruction by applying real-time imaging and tissue fluid measurement tools.

The lymphatic system with its translucent vessel walls and clear fluid still remains under the radar of medical research. Now, with relatively non-invasive imaging technique that allows improved visualisation of the delicate lymphatic capillaries and collectors. This in turn allows us to gain further knowledge of the importance of the lymphatics in health and disease, and of the structure and function of the lymphatic system. This technique, Indocyanine green (ICG) is a fast fluorescence emitting contrast agent ideal for lymphatic investigation due to its ability to quickly bind to plasma proteins (mostly albumin) within the tissue spaces after intradermal injection. Also, its water-soluble properties, rapid excretion and low toxicity make it a safe method to use for lymphatic imaging. Further, its small quantity and intradermal administration using a 30-gauge insulin syringe make it relatively non-invasive.

Commercially available imaging systems for ICG emitted fluorescence are costly and have been challenging to source within Australia. However, it is likely with increased breadth of use and improvements in technology that prices will reduce and access increase. Associated with the cost of commercially available technology, the development of custom made imaging systems has been explored by a number of research groups around the world. The creation of an imaging unit is facilitated on the principles, based on the parameters of ICG excitation and emission wavelengths. In collaboration with the Flinders Biomedical Engineering group we developed a low cost ICG imaging system that was validated with a variety of *ex vivo* and *in vitro* experimental studies including ICG fluorescence intensity standardisation, ICG fluorescence in scavenged porcine material to validate the technique in isolated animal tissues (Chapter 2). This work continued with *in vivo* work in mice, where I tested ICG for its real-time lymphatic vessel tracking ability and exploring quantification methods (Chapter 3).

Clinical human research into ICG lymphatic imaging was also applied in patients who have had severe soft tissue injury after compound fractures (Chapter 5).

Soft tissue injury associated with severe compound lower limb fractures has the potential to cause significant soft tissue damage and disruption to the lymphatic system within, around and distal to the area. The extent of damage to the lymphatic system and subsequent risk of recurrent infections and chronic lymphoedema remained unstudied. The presence of inflammation-induced oedema has been linked to lymphatic dysfunction but the involvement of the lymphatic system has only been raised sporadically in research studies relating to soft tissue injury. Also as the wound healing progresses there is unavoidable scar tissue formation that can further restrict lymphatic flow, impair lymphatic function, and prevent or slow the formation of new lymphatic vessels and pathways.

The onset time of clinically detectable chronic oedema is variable, from immediately after injury to months or even years after the initial injury and apparent recovery. In a lower limb that has suffered the multiple insults of high impact trauma and undergone multiple reconstructions of bone and soft tissue area on each occasion the transport capacity of lymph collectors may be diminished due to scar tissue formation and the need for rerouting and re-cannalisation of the lymphatics.

Utilising ICG and NIR imaging alongside local tissue water, extracellular fluid measurements, general health and lower extremity functioning questionnaires enabled a detailed picture of this at risk lower limb trauma population.

This study has created an improved awareness and understanding of lymphatic disruption and regeneration in post lower limb trauma patients with soft tissue injury and subsequent reconstruction. This knowledge, and the outcomes should improve our detection of the early, perhaps sub-clinical signs of lymphoedema, indicate their risk level and inform further assessment and early intervention to halt or slow the development of further lymphatic changes and thus of lymphoedema.

6.2 Challenges for standardisation of ICG lymphography

ICG lymphography is a novel imaging technique for real-time imaging of superficial lymphatic vessels. It has the potential to be widely implemented in a variety of clinical and research settings. However, it has limitations. The limited penetration depth (1-2cm) means that only superficial lymphatics are visualised, and the range of ICG patterns observed means potential discrepancy between operators and interpretation.

Future research should be focussed on validation of ICG related near-Infrared imaging devices and on strategies for detailed observation of lymphatic patterns observed and evaluation through ICG fluorescence. Ideally, this should be done prior to ICG lymphography

being implementation in clinical practice. This is necessary in order to avoid errors in diagnosis of the general functioning of the superficial lymphatic system and/or misdiagnosis on the presence of chronic oedema or lymphoedema. ICG should also be seen as an *addition* to lymphatic imaging as there is still the deep lymphatic system that is not visualised with ICG, unless in a surgical procedure. Lymphoscintigraphy for example will provide more information on systemic lymphatic failure and the status of the lymph nodes.

The commercially available ICG imaging systems are able to capture emitted fluorescence through the tissues of the area of interest to a depth of 1 to 2cm (reportedly) into the skin (1). Such systems are currently used in clinical, surgical and research settings. However, the high cost associated with these systems and the often unclear exact device specifications (excitation light laser output, LED output or filter specifications) has resulted in research groups constructing their own systems. Many of these systems - including our own - must be investigated and tested to ensure an understanding of all parameters that lead to the ICG fluorescence and its behaviour within the tissue.

Custom made ICG systems' may be cheaper than commercial systems, but they could make it more complicated to reach a consensus due to different operating parameters, image presentation and interpretation. These include differences in working distances from the camera system to skin as well as discrepancies in tissue penetration, which may result in different detection patterns and thus inconsistent diagnoses. Further, different injection depths have also been reported to result in different lymphatic ICG uptake patterns.

Suitable standardised, custom-made ICG systems could potentially be useful in settings such as developing countries that have no access or funding for the commercially available machines. It should be noted that many of the core component materials are individually relatively cheap and their combination and construction relatively easy.

Standardising ICG usages and fluorescence image interpretation in general is a challenge, and will likely remain so. A standard platform for sharing such information with clinicians and researchers (who use ICG for different purposes), would allow us to discuss this challenge in designated consensus meetings. This would be a step toward using ICG lymphography to its full potential.

6.3 The potential quantification of ICG lymphography

Quantifying imaging would improve our understanding of lymphatic physiology. This in turn would improve our knowledge of the aetiology of its pathologies. However, if ICG lymphography is to be implemented as a diagnostic tool for lymphatic vessel functioning, there needs to be a level of quantification implemented. Information that can be obtained from fluorescence imaging can be spatial, such as distances and velocities, or intensity which is concentration based (2). In addition, quantifying fluorescence in biological tissues is relative and dependent on numerous, variable factors; the task of quantification is fundamentally a relative one. Fluorophore binding to proteins, absorption of excitation light, scattering of the fluorescence, biologically variability, camera light saturation levels and camera resolution are all important variable or calibrating factors that can differ between ICG lymphography systems.

In the mice imaging study (Chapter 3) it was evident that lymph node saturation levels can be validated and quantified, provided the ICG fluorescence is within the camera's light detection limits.

Mouse models allowed for a controlled environment and kept biological variability to a minimum. We investigated the use of kymograph analysis for lymphatic imaging quantification. We found that we could observe the rate of ICG absorption by the popliteal lymph node, up to the limit of light saturation, and we were also able to detect lymph vessel

pulsation. If lymph vessel pulsation can be quantified a detailed assessment of lymphatic vessel functioning can be made. This pulsation will vary among individuals but a relative assessment or guideline metric could still be developed. This should be investigated in more detail and cross correlated with other imaging analysis.

6.4 Recruitment database; its importance and challenges

The patient trauma database used in this study has been essential for determining the scope of lymph-related problems and understanding how they affect patients in general. Early and late-term adverse events were identified from the extracted data. However, interrogating and adding to the database proved difficult and highlighted a problematic systemic issue.

Diagnostic coding for compound fractures is based on the Gustilo classification. Often the compound lower leg fracture has more than one code, as it involves other bones (tibia, fibula, malleoli or tibia shaft, fibula shaft) and contains further information about the break fragments themselves (comminuted, segmented or spiral). Therefore, a compound fracture of the lower limb is often represented by a combination of different diagnostic codes, which are not always clearly specified by the specialist.

In addition, procedure coding includes bone fixation as well as soft tissue reconstruction, but does not necessarily consider any other injuries that are usually present in these high energy trauma patients.

When there is a need to extract specific data for research purposes, funding allocation or hospital statistics a complex coding algorithm is required that includes the details of the compound fracture with soft tissue reconstruction, as well as a number of other relevant parameters. This is time consuming and very challenging.

The database accessed in this study specifically reported on treated cases for soft tissue reconstruction, and in fairness, it is a good attempt to maintain consistent accessible information. Full use of this database could not be completed, however, without consulting the medical notes of patients, making for a labour intensive task. The medical notes often were devoid of specific information such as the Gustilo classification, time from accident to ambulance, time from admission to operation, specific details of complication and more importantly information on union and weight-bearing status upon discharge or first follow up.

The difference between bone union and (infected) non-union is a very important outcome measure, determining the long-term morbidity of the patient. But this is also valuable information for the treating specialist which in turn can reflect on the treatment provided and allow the specialist to adjust follow up care so as to achieve the best outcome.

Data on time of specific intervention, such as the initial surgery, wound debridement or final soft tissue coverage, would facilitate prediction analysis that could change and improve standard practice (3). However, obtaining such accurate data with the different coding approaches is problematic.

Investigation of coding discrepancies showed that close to half of the discharge summaries needed to be revised to ensure optimal epidemiological data, and that many data entities were missing. Regular review is recommended in the forms of clinical audit and frequent communication between coders and clinicians. This is crucial for epidemiology data, but also for funding allocation in research that targets improved outcome (4).

The discrepancies and missing entities in the databases' medical records made it difficult to perform any rigorous analysis of demographic statistics, and determine the exact number of people affected by compound tibial fractures, along with their long term outcomes. More

detailed analysis was available from the study sample as there were face to face meetings with the patients, and revision of medical notes and discharge summaries.

With national and international efforts to ensure high quality data in all medical fields, plastic surgery being no exception, high quality evidence is expected and needs to emerge from collected data to inform and resolve clinical controversies. This can only improve patient outcomes and will optimize targeted funding allocation for translational medical research (5).

6.5 Permanent damage of lymphatics after lower limb fractures and wounds

The role of lymphatics in soft tissue wounds and in the subsequent wound healing process has been researched sporadically. Those research projects which do investigate lymph vessels in this context stress the importance of raising awareness of the lymphatic system (6-12). It is an established principle that rupture of the skin and soft tissue will damage and rupture the superficial lymphatics, whose invaluable function is to remove excessive tissue fluid, clear inflammatory mediators and maintain healthy tissue homeostasis. It has also been shown that if lymphangiogenisis is facilitated within a wound, skin cell regeneration will accelerate (13). Given this critical role in wound healing, it is puzzling that lymphatics are consistently neglected in wound healing research and medical textbooks (14, 15).

A similar situation exists regarding bone fractures. Bone union is an important factor, crucial to a lower limb trauma patients' outcome. Union should ideally occur within 3 months of discharge from acute management. The main cause of non-union seems to be infection which is a high risk in the long bones such as the tibia (16, 17). Thus, understanding the role of the lymphatic system in large soft tissue damage is critical, given its pivotal role in the body's immunological response to bacterial invasions.

Szczesny et al. initially studied the response of the lymphatic system to long bone fractures and subsequent healing in dogs (8). They later investigated the same response in humans using lymphoscintigraphy. This revealed enlarged lymph nodes and reduced lymphatic transport in patients affected by lower limb trauma. This study concluded that post trauma patients are at high risk for recurrent infection and chronic lymphoedema (8, 9). Lohrman et al. supported these findings with their lymphography results, observing torturous lymphatic vessels and lymphoedema in patients with extensive lower limb soft tissue trauma (17).

These studies emphasise that poor lymphatic repair, compromised immune system and subsequent infections need to be closely monitored to prevent lymph stasis and progressive development of lymphoedema.

6.6 Reconstructive soft tissue surgery and lymphatic regeneration

Soft tissue reconstruction, after compound lower limb fracture, can be the difference between limb salvage or its amputation, so tissue viability is paramount. Arterial and venous flow within the flap reconstruction is extensively researched and frequently observed and monitored in clinical practice (18-21). However, those publications that identify the regrowth of blood vessels in flap reconstruction pay little or no attention to lymphatic vessel regeneration.

It is known that lymphatic vessels in the dermis tend to regenerate from healthy tissue into the defect. If this does not occur this may well lead to long term lymphatic problems and persistent swelling. With ICG imaging, I did not find any clear re-canalisation between the transferred flap reconstruction and the recipient site surrounding tissue. However, we speculate that a fasciocutaneous flap harvested from areas with well vascularised lymphatic territories such as the medial thigh flap may facilitate bridging the lymphatic gap. With the

vascularisation of the included fascia, this area of harvest may yield more viable lymph vessels and/or greater presence of lymphatic endothelial cells (LEC) than current anterolateral thigh flaps. Bhattacharya et al. identified lymphatic vessels within the deep muscle fascia, suggesting that its incorporation in flap reconstructions may be responsible for a better outcome for flap vascularity (11). More detailed investigation is required before this theoretical assumption can be clinically implemented.

Lymphatic regeneration that penetrates the barriers of scar tissue, skin grafts and burn wounds, has been observed in both human and animal studies, but the viability of regeneration varies with the wound environment. Research suggests that lymphatic endothelial cell (LEC) development remains absent from regenerating tissue for approximately 9-10 days. After this interval, with sufficient repopulation of LECs, the cells then grow together in a vasculogenesic like fashion to form new interconnected lymphatic networks (22). Amann-Vesti et al. examined the application of split thickness skin grafts to chronic venous ulcers and the subsequent lymphatic regrowth. They used fluorescence micro lymphography, on average 71 months after the initial skin grafting. Successful fluorescence micro lymphography was observed in all 15 grafts but only 2 indicated re-established viable lymphatic vessels. The viability was based on the vessel diameter appearance and the apparently normal flow within the vessel (13). The authors concluded that functional micro vascular lymphatic regrowth remains questionable in meshed skin grafting for venous ulcers. It is important to note that chronic venous ulcers have a poor wound environment to start with so therefore poor lymphatic regrowth can be expected, given the knowledge that lymphatics don't usually re-grow in poor perfused environments.

In contrast, in a cohort of deep tissue burns patients, the same authors claimed complete lymphatic regrowth within 4 weeks following the burns. The follow-up at 6 and 18 months

suggested completely normal lymphatic functioning. It seems probable that better debridement in the burns cases was the cause of the improved lymphatic regeneration (14).

These papers highlight the importance of preparation of the wound bed. Preparation is essential for facilitating normalised tissue homeostasis, creating optimal tissue granulation and thus establishing the best possible outcome. In all these processes the lymphatics play a key role.

In reconstructions involving free muscle flaps, vascularised autologous tissue will ensure limb salvage, fracture repair and soft tissue healing. Revascularisation (shown by perfusion studies) indicates a clinically successful outcome. The blood microvasculature within a well vascularised free muscle flap is known to regenerate rapidly (23). However, less information is known regarding the presence of lymphatic vessels within the muscle microcirculation and how they regenerate. In these free flap reconstructions, lymphatic vessels are likely to be disrupted whilst harvesting the flap from the donor site (raising the flap) and when inserting the flap into the defect (24).

Our clinical study suggests that there were no viable superficial lymphatic vessels demonstrated within the muscle flap reconstructions after lower limb injury. This could be attributed to the paucity of existing lymphatic vessels within the muscle flap. It is also likely that scaring on the muscle flap and along its edges may prevent the regrowth of lymphatic vessels from the healthy tissue surrounding the flap, into the flap itself. This hypothesis is supported by Suami et al. who reviewed scarring caused by prior graft surgery in a cadaver leg. Suami et al. explored lymphatic vessel disruption due to the scar and found unusual structured vessels between the scar and the surrounding tissue (25). These vessels were only statically investigated, due it being a cadaveric study, but there were still identifiable differences between scar and previously healthy tissue lymphatic vessel patterns.

In our study, the real-time lymphatic pattern between the free muscle flaps and healthy tissue was unusual compared to the healthy control leg. When compared to the non-affected contralateral limb, the lymphatic vessel morphology seems to have adapted to go around the reconstructed site. Despite this being a significant finding, we still have to question our knowledge of the lymphatic anatomy of the individual since most of our current anatomical information is based on animal studies, human cadavers or anatomical books with drawings that are more than 100 years old. In my study, imaging the lymphatic vessels in real-time in soft tissue reconstructed lower limbs has increased our understanding of the function and adapting ability of the superficial lymphatics.

There have been studies that have reviewed the anatomy of the superficial lymphatic system, and investigated lymphatic recanalization after free flap reconstruction, as well as studies specifically targeting the development of chronic lymphoedema (15). Slavin et al. (1999) researched 8 lower limb participants with free flaps and investigated the functionality of the large lymphatic collectors. Technetium 99m-antimony trisulfide colloid was injected into the flap and visibility of the tracer and time of uptake was noted. The authors concluded that there was a barrier created by the existing scar tissue but normal lymphatic functioning seemed to be restored (5). The difference between the outcome of this study and ours may be due to the type of free flaps used in study of Slavin et al. Slavin et al. investigated reconstructions with musculocutaneous and fasciocutaneous flaps, but those in our study were mostly free muscle flap with skin graft and a free fasciocutaneous flap. The musculocutaneous flap that contains skin, subcutaneous tissue, fascia and muscle should also contain healthy lymphatic vessels and, as mentioned earlier, may thus facilitate reconnection with recipient lymphatic vessels.

6.6.1 Lymphangiogenisis and oedema

We know that interstitial flow and its direction is important to stimulate the vessel formation and cell migration for lymphangiogenesis. Lymphatic endothelial cells (LEC) migrate and organise into lymphatic vessels along the direction of interstitial flow (26).Shear stress forces and hydrostatic pressure continues to stimulate further lymphatic vessel growth to match demand (27).

There is interesting research on natural lymph venous anastomosis (or connections) in free tissue flaps that has relevance here. Using imaging technology, such as lymphoscintigram where a contrast agent suitable for lymphatic transport is injected, the authors were able to observe the contrast agent in the blood before it reached the thoracic duct. This implies natural lymph venous connections (LVC) outside of the subclavian vein (28). LVC have been found distal to the obstruction as a compensatory mechanism (24). It is evident that the venous pressure is crucial for opening LVC, however, levels of venous pressure in flaps are usually unreliable/unpredictable and overall transport volumes remain unknown.

Oedema within the restorative flap is often seen after reconstruction of the lower limbs and relates to post-surgical inflammation. Excessive or prolonged oedema prior to the planned reconstructive surgery, or oedema that arises post-surgery, both compromise the healing process and potentially can cause compression of the venous outflow of the flap. Flap survival is not likely if the venous outflow is compromised. There is limited published human research detailing the interstitial hydrostatic pressure within a reconstructed flap, but it is measured in animal models and proved to be high. A disruption of lymphatic vessels in the dermal plexus and therefore reduction of lymphatic transport caused an increase in interstitial fluid. This can be even further elevated based on increased leakage of intravascular proteins associated with inflammation (29). The local oedema that then occurs within the flap further decreases the capillary filtration as a consequence of increasing intravascular resistance (29).

Further, the longer the oedema persists the more deposition of fat and fibrous tissue occurs (30).

Experience has shown that flaps, mostly free muscle or free fasciocutaneous flaps, can be bulky, and cause discomfort and cosmetic issues for the patient and, in consequence, contouring or flap debulking with liposuction, laser or open excisions are procedures regularly used to address these issues. Fat or adipose tissue infiltrates where atrophy of the unused muscle is apparent in the free muscle flap (31). It should be noted that these debulking procedures run the risk of further damaging lymphatic pathways within the tissues, so perhaps flap debulking should be avoided until we know more about the relationship between lymphatic regeneration and fat tissue (24, 32). Given such complications, it is important we understand precisely where the increased interstitial fluid goes when the lymphatic vessels are not immediately reconnected within lower limb flaps specifically.

6.7 ICG lymphography as a diagnostic tool in lymphatic dysfunction

Despite its limited penetration depth and the lack of standardisation, ICG fluorescence lymphography is now increasingly used as a diagnostic tool for chronic lymphoedema. Unno et al. (2007) were one of the first research groups that explored ICG in patients with lymphoedema. In their follow up study in animals, the authors proposed a quantification method based on the ratio of ICG distance travelled versus time taken to bring standardising objectivity to an otherwise subjective assessment process (18, 19).

One problem with this method, is that the ICG may not travel from point of injection to point of interest only via the superficial lymphatic system, it could travel through the pre-collectors into the collectors towards other locations or past the areas of interest without the operator noticing as it is too deep to detect fluorescence. This could potentially compromise the distance-versus-time metric. Perhaps a method to explore the outline of the lymph drainage

pathways, calculations of vessel diameter, flow dynamics, retrograde and anterograde flow, would be more valuable. These problems notwithstanding, at the conclusion of both the above mentioned studies, the authors recommended ICG usage as a diagnostic tool and emphasised that it is safe to use and less time consuming than comparable methodologies.

With ICG lymphography, patterns of "dermal backflow" are distinctly visible, which means pattern recognition techniques can be applied to explore and classify lymph stasis. Yamamoto et al. (2011) proposed a direct correlation between lymphoedema severity and the ICG patterns in patients with lymphoedema (33). This standard has been adopted by other researchers, however opinions are still divided over the diffuse patterns (12). This is because the diffuse ICG pattern associated with the most severe stage of lymphoedema, can also be observed when the ICG has to penetrate a firm layer of adipose tissue.

Nevertheless, our study demonstrated that ICG lymphography could locate the obstruction in lymph flow superficially, by showing dermal backflow patterns distal of the flap and scar tissue in participants who were referred for further diagnostic imaging. This dermal backflow was also confirmed with lymphoscintigram. Any dermal backflow pattern or non-linear pattern of flow suggests lymphatic failure, as it shows ICG is leaking out of the lymphatic vessels rather than being moved centripetally.

Further experience and publication of findings concerning ICG imaging will undoubtedly increase our understanding of the different ICG fluorescence patterns, and of their uniqueness for each individual.

6.8 Post trauma physical and mental outcome studies

The quality of life outcome after a traumatic experience directly impacts upon the long-term physical wellbeing of the person affected. In addition to the physical outcome, after lower limb trauma and reconstruction, the mental health of the patient must not be overlooked.

Many factors have been identified that contribute to a patient's overall outcome, including, but not limited to, the ability to return to work, costs of health care and rehabilitation and post-operative complications, whether immediate or latent. These factors are recognised as playing a role in the overall mental coping with the trauma (34). Numerous general quality of life questionnaires has been developed over the last two decades focussing on physical and mental health with an increased interest in patient centred outcomes.

There exists substantial research examining both the functional and the psychological status of patients after major lower limb trauma and reconstruction. In general, the long term outcome of such patients seems poor, commonly featuring chronic disability, loss of function of the affected limb, ongoing pain, recurrent infection and hospitalisation (35-37). The clinical outcomes obtained through our research are similar.

Although all participants in our research reported swelling, it did not seem to affect them in their daily lives as much as the pain and the loss of function. This absence of concern could be a problem, as it may increase the risk of behaviour or accidents that aggravate the condition, causing intermittent swelling to develop into lymphoedema, which has a recognised significant impact on a person's daily life (38).

6.9 Limitations of the study

Three limitations must be noted.

First: The lymphatic imaging in this study was performed with the custom made imaging system, comprehensive video and still images were obtained. To progress such a custom made system to diagnostic situations, cross-validated with commercial ICG imaging system (e.g Hamamatsu Photo Dynamic Eye) should occur.

Second: The sample size for the clinical trial was smaller than ideal. Many of the patients contacted to participate in the study were either lost prior to follow up or were from rural/remote areas within South Australia or even interstate. A large number of people opted out, preferring not to volunteer in a study that would not be directly beneficial for them, or declined due to physical or mobility limitations caused by their accident. Some of these patients even mentioned in phone conversation that they were still traumatised by the accident and did not feel ready to come to the hospital for the study. Finally, some patients were still engaged in legal battles with insurance companies and received legal advice not to be assessed by anyone connected with the hospital.

Third: The mice studies were aimed at assessing the baseline utility of ICG in small animal research. Further study is required to better characterise the use in ICG in a wider range of animal (disease) models.

6.10 Overall conclusion

This study contributes to the knowledge of lymphatic imaging, lymphatic vessel structure and functioning in and around lower limb soft tissue reconstructions following severe high impact trauma.

Specifically, this research demonstrates that:

1. ICG lymphography is a feasible, relatively simple non-invasive technique for imaging the superficial lymphatic system.
2. ICG real-time imaging can be used in animal models.
3. ICG lymphography is useful for assessing post lower limb Gustilo IIIB trauma patients with subsequent soft tissue reconstruction. In particular, we observed that superficial lymphatic vessels were not visible in the lower limb free muscle flap reconstructions and that lymphatic vessels had an altered pathway which avoided scar tissue. The control limb

also showed faster uptake of the ICG dye compared to the affected limb. In the local reconstruction as well as the free muscle flap reconstruction group it was evident that scar tissue blocked the continuous flow of superficial lymphatics and seemed to caused local dermal backflow patterns. There were no viable or normal lymphatics within the free muscle or musculocutaneous flap. In all cases there was a block to lymph flow from the surrounding tissues, along the scar tissue edge. Lymphatic function in patients with lower limb trauma is negatively affected. Long term or 12 month follow up in patients shows no change in the lymphatic flow or fluid in the leg.

4. Lower limb trauma patients are at risk for recurrent infections, subsequent lymphoedema, and a reduced quality of life. All affected limbs seem to have significant higher extracellular fluid, high local tissue fluid and larger limb volumes.

6.11 Recommended directions for future research

I have several recommendations that stem from the findings in this thesis.

1. Establish consensus for ICG injection-imaging system.
2. More research on quantifiable methods for ICG lymphography is needed.
3. More flaps and flap types must be researched in detail and compared to each other in terms of the impact on the lymphatic pathways development, lymphatic function and the presence of lymphedema. The flaps to include and perhaps to compare are: free muscle flap, free fasciocutaneous flap, local hatchet flap, and local fasciocutaneous flap.

References

1. Marshall M, Rasmussen J, Tan C, Aldrich M, Adams K, Wang X, et al. Near-Infrared fluorescence imaging in humans with indocyanine green: A review and update. The Open Surgical Oncology Journal. 2010;2(2):12-25.

2. Waters JC. Accuracy and precision in quantitative fluorescence microscopy. The Journal of cell biology. 2009;185(7):1135-48.

3. Pollak AN, Jones AL, Castillo RC, Bosse MJ, MacKenzie EJ, Leap Study Group. The relationship between time to surgical debridement and incidence of infection after open high-energy lower extremity trauma. The Journal of Bone and Joint Surgery. 2010;92(1):7-15.

4. Chin N, Perera P, Roberts A, Nagappan R. Review of medical discharge summaries and medicaldocumentation in a metropolitan hospital: impact ondiagnostic-related groups and Weighted Inlier Equivalent Separation. Internal Medicine Journal. 2013;43(7):767-71.

5. Fan KL, Patel KM, Mardini S, Attinger C, Levin LS, Evans KK. Evidence to support controversy in microsurgery. Plastic and reconstructive surgery. 2015;135(3).

6. Khazanchi R, Rakshit K, Bal C, Guleria S, Sinha S, Srivastava A. Evaluation of lymphatic drainage in free flaps by lymphoscintigraphy: a preliminary study. Br J Plast Surg. 1996;49(2):123-8.

7. Slavin SA, Upton J, Kaplan WD, Van den Abbeele AD. An investigation of lymphatic function following free-tissue transfer. Plast Reconstr Surg. 1997;99(3):730-41; discussion 42-3.

8. Szczesny G, Olszewski WL. The pathomechanism of posttraumatic edema of lower limbs: I. The effect of extravasated blood, bone marrow cells, and bacterial colonization on tissues, lymphatics, and lymph nodes. Journal of Trauma. 2002;52(2):315-22.

9. Szczesny G, Olszewski WL. The pathomechanism of posttraumatic edema of the lower limbs: II--Changes in the lymphatic system. Journal of Trauma. 2003;55(2):350-4.

10. Lohrmann C, Pache G, Felmerer G, Földi E, Schaefer O, Langer M. Posttraumatic edema of the lower extremities: evaluation of the lymphatic vessels with magnetic resonance lymphangiography. Journal of vascular surgery. 2009;49(2):417-23.

11. Bhattacharya V, Barooah PS, Nag TC, Chaudhuri GR, Bhattacharya S. Detail microscopic analysis of deep fascia of lower limb and its surgical implication. 2010.

12. Meier TO, Guggenheim M, Vetter ST, Husmann M, Haile SR, Amann-Vesti BR. Microvascular regeneration in meshed skin transplants after severe burns. Burns Journal. 2011;37(6):1010-4.

13. Marino D, Luginbühl J, Scola S, Meuli M, Reichmann E. Bioengineering dermo-epidermal skin grafts with blood and lymphatic capillaries. Science translational medicine. 2014;6(221).

14. Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. Journal of Internalional Medical Research. 2009;37(5):1528-42.

15. Ireton JE, Unger JG, Rohrich RJ. The role of wound healing and its everyday application in plastic surgery: a practical perspective and systematic review. Journal of Plastic and Reconstructive Surgery. 2013;1(1).

16. Bell A, Templeman D, Wienlein JC. Nonunion of the Femur and Tibia: An Update. The Orthopedic clinics of North America. 2016;47(2):365-75.

17. Willey M, Karam M. Impact of Infection on Fracture Fixation. Orthopedic Clinics of North America. 2016;47(2):357-64.

18. Kraemer R, Lorenzen J, Knobloch K, Papst S, Kabbani M, Koennecker S, et al. Free flap microcirculatory monitoring correlates to free flap temperature assessment. Journal of Plastic and Reconstructive Aesthetic Surgery. 2011;64(10):1353-8.

19. Hou Z, Irgit K, Strohecker KA, Matzko ME, Wingert NC, DeSantis JG, et al. Delayed flap reconstruction with vacuum-assisted closure management of the open IIIB tibial fracture. Journal of Trauma. 2011;71(6):1705-8.

20. Vaienti L, Gazzola R, Benanti E, Leone F, Marchesi A, Parodi PC, et al. Failure by congestion of pedicled and free flaps for reconstruction of lower limbs after trauma: the role of negative-pressure wound therapy. J Orthop Traumatol. 2013;14(3):213-7.

21. Starnes-Roubaud MJ, Peric M, Chowdry F, Nguyen JT, Schooler W, Sherman R, et al. Microsurgical Lower Extremity Reconstruction in the Subacute Period: A Safe Alternative. Journal of Plastic and Reconstructive Surgery. 2015;3(7).

22. Rutkowski JM, Boardman KC, Swartz MA. Characterization of lymphangiogenesis in a model of adult skin regeneration. American journal of physiology. 2006;291(3):1402-10.

23. Shandalov Y, Egozi D, Koffler J, Dado-Rosenfeld D, Ben-Shimol D, Freiman A, et al. An engineered muscle flap for reconstruction of large soft tissue defects. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(16):6010-5.

24. Miranda Garcés M, Mirapeix R, Pons G, Sadri A, Masià J. A comprehensive review of the natural lymphaticovenous communications and their role in lymphedema surgery. Journal of Surgical Oncology. 2016;113(4):374-80.

25. Suami H, Pan WR, Taylor GI. The lymphatics of the skin filled by a dermal backflow: an observation in a scarred cadaver leg. Journal of Lymphology. 2007;40(3):122-6.

26. Boardman KC, Swartz MA. Interstitial flow as a guide for lymphangiogenesis. Journal of Circulation Research. 2003;92(7):801-8.

27. Schwartz MA, Simons M. Lymphatics thrive on stress: mechanical force in lymphatic development. The European Molecular Biology Journal. 2012;31(4):781-2.

28. Heymans O, Fallais C, Hustinx R. Intratissular lymphaticovenous anastomoses demonstrated by perioperative intramuscular injection of 99mTC-colloids. Lymphatic research and biology. 2006;4(1):29-33.

29. Gaboriau HP, Murakami CS. Skin anatomy and flap physiology: Management of facial cutaneous defects, Part 1 Journal of Otolaryngologic Clinics of North America. 2001;34(3):555-69.

30. Harvey NL. The Link between Lymphatic Function and Adipose Biology. Annals of the New York Academy of Sciences. 2008;1131(01):82-8.

31. Salmi A, Tukiainen E, Härmä M, Asko-Seljavaara S. A prospective study of changes in muscle dimensions following free-muscle transfer measured by ultrasound and CT scanning. Journal of Plastic and Reconstructive Surgery. 1996;97(7):1443-50.

32. Ooi A, Wong CH, Ong YS. Combined use of liposuction and arthroscopic shaver in lower-limb fasciocutaneous flap contouring. Journal of Plastic, Reconstructive & Aesthetic Surgery. 2013;66(4):538-42.

33. Yamamoto T, Narushima M, Doi K, Oshima A, Ogata F, Mihara M, et al. Characteristic indocyanine green lymphography findings in lower extremity lymphedema: the generation of a novel lymphedema severity staging system using dermal backflow patterns. Journal of Plastic and Reconstructive Surgery. 2011;127(5):1979-86.

34. Burton M, Walters SJ, Saleh M, Brazier JE. An evaluation of patient-reported outcome measures in lower limb reconstruction surgery. Journal of the International Society of Quality of Life Research. 2012;21(10):1731-43.

35. Higgins TF, Klatt JB, Beals TC. Lower Extremity Assessment Project (LEAP)--the best available evidence on limb-threatening lower extremity trauma. The Orthopedic clinics of North America. 2010;41(2):233-9.

36. Hoogendoorn JM, van der Werken C. Grade III open tibial fractures: functional outcome and quality of life in amputees versus patients with successful reconstruction. Journal of Injury. 2001;32(4):329-34.

37. Bosse MJ, MacKenzie EJ, Kellam JF, Burgess AR, Webb LX, Swiontkowski MF, et al. An analysis of outcomes of reconstruction or amputation after leg-threatening injuries. The New England journal of medicine. 2002;347(24):1924-31.

38. Armer JM, Hulett JM, Cormier JN, Stewart BR, Wanchai A, Cromwell KD. Lymphedema and its impact on quality of life. In: Neligan PC, Masai J, Piller NB, editors. Lymphedema Complete medical and surgical management. 1. USA: Taylor and Francis Group; 2016. p. 24-41.

Appendix A

Imaging system cost breakdown

Equipment	Specifications	Cost (in AUS dollar - 2013)
Eight laser flashlights	Pulsar , L-808S laser diode Wavelength: 780nm Lens diameter: 22mm Equivalent IR Power: 250mW Laser Class: Class 1 Range of power adjustment (min/max): 125-250mW Range of beam divergence: 4.5-7° Custom supply made (6V, 1.5 A nominal)	$2000 ($250 each)
Video Camera	Panasonic, B/W WV-BP330 Series camera 1/3" CCD Scanning Area: 4.9 (H) x 3.7 (V) mm Scanning System: 2:1 interlace Video Output 1.0V[p-p] EIA composite 75W/BNC connector	No cost- Department Asset
Aspherical IR Varifocal Lens	Daiwon Optical Co. Ltd, VIR3080AS Focal Length: 3.0-8.0mm Viewing Angle: 44.1°-118.7° diagonal Dimensions: 35mmØx48mm(L)	No cost- Department Asset
Long pass filter	Edmund Optics Pty Ltd, LP 850nm Rejection Wavelength: 200-835nm Transmission Wavelength : 865-1650nm Diameter : 12.5mm Transmission: ≥ 91% average Cut-On Wavelength : 850nm Cut-On Tolerance: ± 1%	$213.10
Video Capture Software	VirtualDub Video capture/processing utility for 32 & 64-bit Windows platforms	Free software
Portable light source	Battery operated Wide wavelength spectrum	No cost- Department Asset
Trolley and six linear bearings	Standard hospital push trolley	Trolley on loan- Bearings ~$480 ($80 each)
Total costs		$2693.10

Appendix B

Medical history form

Government of South Australia
Central Northern Adelaide Health Service

Flinders UNIVERSITY

Local and general lymphoedema incidence following lower limb soft tissue trauma and subsequent reconstructive surgery.

Ms Malou van Zanten, Professor Neil Piller, Mr Yugesh Caplash, Dr Raakhi Mistry, Dr Andrew Campbell-Lloyd, Dr James Finkemeyer,

Lymphoedema Assessment Short Medical History form

Date:
Time:
Assessor Initials:

ALLOCATED SUBJECT ID:

Participant Demographics

Name:
DOB:
Address:
Contact number:

Lower leg trauma

Mechanism of impact
Leg affected
Type of fracture
Other injuries
Date of reconstructive surgery
Type of reconstruction
Events during healing
Other relevant issues

Current medication

_____

_____

☐ **Checked medication list for possible interaction?**

Psychosocial/functional status

☐ Social support
☐ Employed

Type of employment: (eg sitting or standing)
Mobility
Activities of daily living

Pain assessment

☐ Any pain at present
☐ Any current treatment for pain
- Type of treatment or medication:_____

Pain located
Pain character and pain score 0-10

Oedema history

Dominant arm L / R	Dominant leg L / R
Affected leg L / R	
Any swelling at present?	YES / NO
Location swelling	
Started	
Caused?	
Ever had an infection of the leg?	YES / NO

☐ Treatment for the oedema?
- Type

☐ Compression?
- Type

Any swelling in the past	YES / NO
Location swelling	
Started	
Resolved how and when?	

- ☐ Is the swelling worse in the evening but resolves in the morning?
- ☐ Is the swelling worse when it is hot?
- ☐ Is the swelling only in the reconstructed site?
- ☐ Is the swelling only below the reconstructed site?
- ☐ Is the swelling in the whole leg?

Clinical presentation (*Tick if applies*)

- ☐ Positive stemmer sign
- ☐ Pitting
- ☐ Location of pitting test……
- ☐ Poor skin condition (dry scaling skin)
- ☐ Mild scar tissue reconstructed site
- ☐ Severe scar tissue reconstructed site
- ☐ Consistency of flap
  - Firm
  - Fibrotic
  - Soft
  - Pitting

- ☐ Blood pressure _____Pulse_____
- ☐ Weight _____
- ☐ Height _____

To avoid unwanted interaction with ICG please answer the following:

- ☐ Any thyroid dysfunction?
- ☐ Any renal failure or other kidney dysfunction?
- ☐ Any liver failure or other dysfunction?
- ☐ Do you suffer from epilepsy seizures? Ever had an epilepsy seizure?
- ☐ Any upcoming test or have you had tests done in the last 6 months that involved testing liver function, kidney function or thyroid function (radio-active iodine uptake)?
- ☐ To the best of your knowledge have you ever had an allergic reaction to ICG?
- ☐ To the best of your knowledge do you have an allergy for iodine of shell fish?

Appendix C

Health Questionnaire

SF-12® **Participant ID:**
Date:

The information from this health questionnaire will help us understand how you feel and how well you are able to do your usual activities. Answer every question by placing a check mark in the box in front of the appropriate answer. If you are unsure about how to answer a question, please give the best answer you can and make a written comment beside your answer. Don't hesitate to ask the researcher if you need help to complete the form.

1. In general, would you say your health is:
- ☐ Excellent (1)
- ☐ Very Good (2)
- ☐ Good (3)
- ☐ Fair (4)
- ☐ Poor (5)

The following two questions are about activities you might do during a typical day. Is your health now limiting you in these activities? If so, how much?

2. Moderate activities such as moving a table, pushing a vacuum cleaner, bowling, or playing golf:
- ☐ Yes, Limited A Lot (1)
- ☐ Yes, Limited A Little (2)
- ☐ No, Not Limited At All (3)

3. Climbing several flights of stairs:
- ☐ Yes, Limited A Lot (1)
- ☐ Yes, Limited A Little (2)
- ☐ No, Not Limited At All (3)

During the past **4 weeks** have you had any of the following problems with your work or other regular activities as a result of your physical health?

4. Accomplished less than you would like:
- ☐ Yes (1)
- ☐ No (2)

5. Were limited in the kind of work or other activities:
- ☐ Yes (1)
- ☐ No (2)

During the past **4 weeks**, were you limited in the kind of work you do or other regular activities as a result of any emotional problems (such as feeling depressed or anxious)?

6. Accomplished than you would like:
- ☐ Yes (1)
- ☐ No (2)

SF-12® **Participant ID:**
Date:

7. Didn't do work or other activities as carefully as usual:
- ☐ Yes (1)
- ☐ No (2)

8. During the past **4 weeks**, how much did pain interfere with your normal work (including both work outside the home and housework)?
- ☐ Not At All (1)
- ☐ A Little Bit (2)
- ☐ Moderately (3)
- ☐ Quite A Bit (4)
- ☐ Extremely (5)

The next three questions are about how you feel and how things have been during the past **4 weeks**. For each question, please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past **4 weeks:**

9. Have you felt calm and peaceful?
- ☐ All of the Time (1)
- ☐ Most of the Time (2)
- ☐ A Good Bit of the Time (3)
- ☐ Some of the Time (4)
- ☐ A Little of the Time (5)
- ☐ None of the Time (6)

10. Did you have a lot of energy?
- ☐ All of the Time (1)
- ☐ Most of the Time (2)
- ☐ A Good Bit of the Time (3)
- ☐ Some of the Time (4)
- ☐ A Little of the Time (5)
- ☐ None of the Time (6)

11. Have you felt downhearted and blue?
- ☐ All of the Time (1)
- ☐ Most of the Time (2)
- ☐ A Good Bit of the Time (3)
- ☐ Some of the Time (4)
- ☐ A Little of the Time (5)
- ☐ None of the Time (6)

12. During the **PAST 4 WEEKS,** how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?
- ☐ All of the Time (1)
- ☐ Most of the Time (2)
- ☐ A Good Bit of the Time (3)
- ☐ Some of the Time (4)
- ☐ A Little of the Time (5)
- ☐ None of the Time (6)

Appendix D

Lower Extremity Functional Scale

Lower Extremity Functional Index

We are interested in knowing whether you are having any difficulty at all with the activities listed below because of your lower limb problem for which you are currently seeking attention. Please provide an answer for each activity.

Today, do you or would you have any difficulty at all with:

(Circle one number on each line)

Activities	Extreme Difficulty or unable to perform activity	Quite a bit of difficulty	Moderate difficulty	A little bit of difficulty	No difficulty
a. Any of your usual work, housework or school activities.	0	1	2	3	4
b. Your usual hobbies, recreational or sporting activities	0	1	2	3	4
c. Getting into or out of the bath.	0	1	2	3	4
d. Walking between rooms.	0	1	2	3	4
e. Putting on your shoes or socks.	0	1	2	3	4
f. Squatting.	0	1	2	3	4
g. Lifting an object, like a bag of groceries from the floor.	0	1	2	3	4
h. Performing light activities around your home.	0	1	2	3	4
i. Performing heavy activities around your home.	0	1	2	3	4
j. Getting into or out of a car.	0	1	2	3	4
k. Walking 2 blocks.	0	1	2	3	4
l. Walking a mile.	0	1	2	3	4
m. Going up or down 10 stairs (about 1 flight of stairs).	0	1	2	3	4
n. Standing for 1 hour.	0	1	2	3	4
o. Sitting for 1 hour.	0	1	2	3	4
p. Running on even ground.	0	1	2	3	4
q. Running on uneven ground.	0	1	2	3	4
r. Making sharp turns while running fast.	0	1	2	3	4
s. Hopping.	0	1	2	3	4
t. Rolling over in bed.	0	1	2	3	4
COLUMN TOTALS					

Score variation ± 6 LEFTS points
MDC & MCID = 9 LEFS points

Score _____/80

Appendix E

Author contribution list

Chapter 1 Introduction to thesis and literature review (from paragraph 1.8 -1.18) is based on peer reviewed publication:

Van Zanten M, Piller N, Finkemeyer J, Caplash Y. 'A review of severe lower limb trauma with extensive soft tissue loss and subsequent reconstructive surgery: its impact on the lymphatic system'. Journal of Australian Wound Management Association. Wound Practice and Research 22(2). 2013

MvZ, NP, JF are the main authors of the manuscript, design and initiation. NP, YC reviewed, and co-authored the manuscript.

And additional peer reviewed ISL conference proceeding paper:

Van Zanten M, Caplash Y, Campbell-Lloyd A, Mistry R, Finkemeyer J, Piller N. 'Local and general lymphoedema incidence in severe leg trauma with extensive soft tissue loss; Measurement of lymphatic repair'. Progress in Lymphology, Proceedings of the 24th conference of the International Society of Lymphology. Lymphology 47 (suppl) 36-40. 01/2014.

MvZ, YC, RM are the main authors of the manuscript, design and initiation. JF, ACL, NP, YC reviewed, and co-authored the manuscript.

Chapter 2 Imaging the lymphatic system (from paragraph 2.3-2.5) is based on peer reviewed publication:

Van Zanten M*, Pallotta O*, McEwen M, Burrow L, Beesley J, Piller N. 'The development and validation of a custom made Indocyanine Green Fluorescence Lymphatic Vessel Imager' Journal of Biomedical Optics. 20(6) 1-6 2015.

MvZ, OP *contributed equally to the work. MvZ, OP are the main authors of the manuscript, designed and initiated the project, MMc, LB, JB, NP helped design the project, reviewed, and co-authored the manuscript

Chapter 4 materials and methods (paragraph 4.2.2) is based on peer reviewed publication:

Van Zanten M, Piller N, Ward L C. 'Inter-changeability of impedance devices for lymphoedema assessment'. Lymphatic Research and Biology. Ahead of print Nov 2015. Published in print: June 2016, 14(2): 88-94. doi:10.1089/lrb.2015.0026

Author contributions: MvZ and LCW were the main authors of the manuscript, initiated and supervised the project, and performed statistical analysis and interpretation. NP helped design the project, reviewed, and co-authored the manuscript.

Chapter 5 Human results (paragraph 5.7) and Chapter 6 Discussion and conclusion (paragraph 6.2-6.3-6.6-6.7) are based on:

Van Zanten M, Mistry R, Suami H, Campbell-Lloyd A, Finkemeyer J, Piller N, Caplash Y. "The lymphatic response to injury with soft tissue reconstruction in high-energy open tibial fractures of the lower extremity". Journal of Plastic and Reconstructive Surgery (PRS). *Accepted July 2016. In press.*

Authors contributions: MvZ, RM and HS were the main authors of the manuscript, initiated and designed the project, collected data and image analysis. JF, ACL, NP, YC helped design the project, supervised, reviewed and co-authored the manuscript.

Additional publications within candidature

Mistry R, Forster N, Neuhaus S.J, **van Zanten M**, Jeeves A, Caplash Y. 'Indocyanine Green Lymphography Following Axillary Lymph Node Dissection'. Journal of Lymphoedema. Vol 10(1) pp20-24 2015.

Van Zanten M, van den Dungen D, Rienstra C, Kielstra – Oppenhuizen N, Stienstra P. 'The role of skin therapists in the Netherlands – maintaining skin health in lymphoedema'. Research and audit - Journal of Lymphoedema. Vol 9 (1) 2014

Hoelen W, **van Zanten M** , Bosman J. 'Preliminary Results of a Prospective Controlled Study to Determine the Use of Ultrasound as a Diagnostic Tool in Lipoedema' International Lymphoedema Framework Conference Paper, Glasgow, Scotland. 2014

Van Zanten M, 'Breast Oedema: diagnosis, treatment and management' Under Pressure 3M Compression Therapy Australasian Newsletter, 2, P5. 2014

Van Zanten M, Kean B. 'Use of a two-layer compression system in severe bilateral leg lymphoedema with ulceration: A case report' Journal of Lymphoedema. Vol (2).P24/26. 2013

Presentations within candidature 2012-2016

Van Zanten M. Imaging the lymphatic vessels in reconstructed lower limbs with indocyanine green. Australasian Lymphology Association and International Lymphoedema Framework Conference. Darwin , Australia. 06/2016

Van Zanten M. The risk for lymphoedema in lower limb trauma with extensive soft tissue loss. Australasian Lymphology Association and International Lymphoedema Framework Conference. Darwin , Australia. 06/2016.

Van Zanten M. Imaging superficial lymphatics after soft tissue trauma. PhD final seminar. Flinders University School of Medicine South Australia. 12/2015

Van Zanten M. Lymphatic response following soft tissue reconstruction of compound lower limb fractures. PhD Student Day. Flinders University School of Medicine South Australia. 10/2015

Van Zanten M. Lymphatic response following soft tissue reconstruction of compound lower limb fractures. World Lymphology congress/ International Society of Lymphology, San Francisco, USA 09/2015

Van Zanten M. Increased risk for the development of lymphedema in lower limb trauma with extensive soft tissue loss. World Lymphology International Society of Lymphology congress, San Francisco, USA.09/2015

Van Zanten M. ICF core set development for lymphoedema in South Australia International Society of Lymphology congress, San Francisco, USA 09/2015

Van Zanten M. 'Regeneration and imaging of lymphatics following soft tissue damage' Cancer Council South Australia. Adelaide, Australia . 05/2015

Van Zanten M. 'Measurement of lymphatic repair: Local and general lymphoedema incidence in severe leg trauma with extensive soft tissue loss' Victoria University Melbourne. Dermal Sciences Students. 05/2015

Van Zanten M. 'Esther Bright Travel Award winner 2012. What has happened since then?' Soroptimist International. Adelaide, Australia. 04/2015

Van Zanten M. 'The impact of severe lower leg trauma with extensive soft tissue loss on the lymphatic system' The International Lymphoedema Framework Conference, Glasgow, Scotland.06/2014

Van Zanten M. "ICF South Australia" Consensus Meeting International Lymphoedema Framework Pre-Conference, Glasgow, Scotland 06/2014

Van Zanten M. ' Local and general lymphoedema incidence in severe leg trauma with extensive soft tissue loss; Measurement of lymphatic repair' 24th conference International Society of Lymphology, Rome Italy. 09/2013

Van Zanten M. 'The importance of skin and scar tissue assessment in post melanoma patients' The International Lymphoedema Framework Conference. Montpellier, France. 06/2012

Van Zanten M. 'The importance of skin and scar tissue assessment in post melanoma patients' The Australasian Lymphology Association. Cairns, Australia. 05/2012

Poster Presentations 2012-2016

Van Zanten M, Mistry R, Suami H, Campbell-Lloyd A, Finkemeyer J, Piller N, Caplash Y. 'Lymphatic Response Following Soft Tissue Reconstruction of Gustillo 3b Tibial Fractures'. E-poster Plastic Surgery Congress. Brisbane, Australia.05/2015

Van Zanten M, Mistry R, Campbell-Lloyd A, Finkemeyer J, Piller N, Caplash Y. 'Lymphatic system response to severe lower limb trauma'. E-Poster. Plastic Surgery Congress - The Meeting. Washington, USA. 10/2014.

Awards and funding within candidature

- Flinders University Research Student Travel award to attend and present at the International Society of Lymphology meeting 2015 ($500).
- Lymphoedema Support Group South Australia to attend and present at the International Lymphoedema Framework meeting 2014 ($ 500).
- Best day presenter International Lymphoedema Framework meeting 2014 (http://blogs.flinders.edu.au/flinders-news/2014/07/16).
- PhD student publication award 2014 ($ 500).
- 3M Coban 2 Compression Travel Grant to attend and present Australasian Lymphology Association meeting 2014 (€1500).
- The Lymphology Presidential Prize / International Society of Lymphology 2013 (US $ 600).
- Flinders University Innovative Research Grant 2012 ($5000).
- Soroptimist International Esther Bright Travel Award to attend and present at the International Lymphoedema Framework meeting 2012 ($1000).
- Flinders Volunteers Conference registration for the International Lymphoedema Framework meeting 2012 ($ 240).
- Flinders Foundation Travel Award to attend and present at Australasian Lymphology Association meeting 2012 ($1500).

Appendix F

Listed peer reviewed publications

A review of severe lower limb trauma with extensive soft tissue loss and subsequent reconstructive surgery: its impact on the lymphatic system

Malou van Zanten, Neil Piller, James Finkemeyer & Yugesh Caplash

ABSTRACT

Lymphoedema is the accumulation of fluid in the tissues. Higher cytokine levels within this fluid can cause chronic inflammation, which leads to poor tissue health and repair. As lymphatic failure progresses, lymphoedema worsens, resulting in visible swelling and mobility issues. These issues are associated with discomfort, heaviness and pain. Lymphoedema is more commonly recognised as secondary to cancer and its treatment; however, it can also occur after trauma with extensive soft tissue damage or loss. Severe open fractures due to high-energy trauma require soft tissue reconstruction with local, regional or free tissue in addition to the fixation of bony injury. Oedema, both within and surrounding the reconstructed site, can present acutely in the post-surgery setting. However, in some patients the swelling fails to resolve and the patient develops chronic oedema. This is lymphoedema, when the lymphatic system is in a state of failure, either due to its inability to regenerate in the wounded area or its inability to handle the increased load imposed in the post-traumatic period. Findings in this literature review show no current best practice protocols are available at this stage of traumatic lower limb lymphoedema. However, new lymphatic imaging techniques focused on lymphatic function may provide a better understanding of lymphatic failure, possibly identifying the reasons for poor regrowth and inosculation of lymphatic channels, or the effect of increased loads on the existing system. This literature review is in preparation for a clinical study.

Keywords: Lymphoedema, lower limb trauma, reconstruction, lymphatic regeneration, lymphatic imaging.

Ms Malou van Zanten*
BSc Health, MSc Candidate
Lymphoedema Research Unit, Level 3, Department of Surgery, Flinders Medical Centre, Flinders University of South Australia, Bedford Park, SA 5042
Email malou.vanzanten@flinders.edu.au

Professor Neil Piller
BScHons, PhD
Lymphoedema Research Unit, Level 3, Department of Surgery, Flinders Medical Centre, Flinders University of South Australia, Bedford Park, SA 5042
Email neil.piller@flinders.edu.au

Dr James Finkemeyer
BBiomedSci, BMBS
Department of Plastic & Reconstructive Surgery
Level 5, Department of Surgery, Royal Adelaide Hospital
Adelaide, SA, 5000
Email james.finkemeyer@wh.org.au

Yugesh Caplash
Director, Department of Plastic and Reconstructive Surgery

* Corresponding author

THE LYMPHATIC SYSTEM

The lymphatic system is vital for tissue homeostasis and relies on the tentative balance between the entry of lymphatic fluid from tissue and the ability of the system to transfer that fluid centrally. The lymphatic system consists of a deep system, the lymph collectors, and a superficial system, the lymph pre-collectors and capillaries. These two systems are separated by the deep fascia. In the event of deep system failure, fluids and their contents are re-routed via the superficial system. This re-routing, however, can be affected by induration of the fascia and of the epi-fascial tissues in general. Unlike the vascular system, the lymphatic system is not a closed circulatory system. This means lymph can be transported from distal to proximal areas, and also from the deep system to the superficial system and vice versa. A vital characteristic of the lymph capillaries is that they are highly permeable[1,2]. The lymphatic system is responsible for transporting proteins, fluid, macromolecules, inflammatory mediators such as cytokines, fatty acids and immunological cells which plays an important role in both the regulation of homeostasis and immune responses[3]. The major lymph collectors are often situated within the adventitia of large arteries and veins. Lymphatic flow in peri-arterial lymphatics is induced by the arterial pulsatile flow[4]. As lymphatic fluid is slowly transported through the body it enters lymph nodes.

LOCAL AND GENERAL LYMPHŒDEMA INCIDENCE IN SEVERE LEG TRAUMA WITH EXTENSIVE SOFT TISSUE LOSS; MEASUREMENT OF LYMPHATIC REPAIR

Malou van Zanten [1]*, Yugesh Caplash [2], Andrew Campbell-Lloyd [3], James Finkemeyer [4], Neil Piller [5].

[1] PhD candidate, Department of Surgery, Flinders University of South Australia, Adelaide, Australia

[2] Head of Department Plastic and Reconstructive Surgery Royal Adelaide Hospital, Adelaide, Australia

[3] Clinical and Research Registrar, Plastic and Reconstructive Surgery Royal Adelaide Hospital, Adelaide, Australia

[4] Clinical and Research Registrar Plastic and Reconstructive Surgery Royal Adelaide Hospital, Adelaide, Australia

[5] Director Lymphoedema Research Unit, Department of Surgery, Flinders University of South Australia, Adelaide, Australia

(*) Corresponding author: vanz0017@uni.flinders.edu.au

For the full text article please refer to the Wound Practice & Research Journal of the Australian Wound Management Association, June 2013, Vol. 21(2), p. 66-68.

ABSTRACT

Lymphoedema is the accumulation of fluid in the tissues. Higher cytokine levels within this fluid can cause chronic inflammation which leads to poor tissue health and repair. Lymphoedema can occur secondary due to high energy trauma with extensive soft tissue loss. Severe open fractures require soft tissue reconstruction with local, regional or free tissue in addition to the fixation of bone. Oedema, both within and surrounding this reconstructed site can present acutely in the post-surgery setting but in some cases the swelling fails to resolve and the patient develops chronic (lymph) oedema. The lymphatic system is in failure, either due to its inability to regenerate within or across the reconstructed area or its inability to handle the increased load imposed on it during the post-traumatic period.

There is no current best practice protocol available to manage lower limb lymphoedema following trauma. Research shows there is poor long term follow up for these post trauma patients and lymphoedema is not a parameter of interest in any outcome studies. Lymph flow in these reconstruction flaps has been reported but not measured accurately. New functional lymphatic imaging techniques involves the use of fluorescence contrast agent Indocyanine Green (ICG) which has proven to be safe, cost effective and accurate. This contrast agent binds to proteins and therefore can give detailed pattern of superficial lymphatic vessels. Of interest are those vessels within the reconstructed areas and across scar tissue borders to normal tissue.

Journal of Biomedical Optics 20(6), 066003 (June 2015)

Development and validation of a custom made indocyanine green fluorescence lymphatic vessel imager

Olivia J. Pallotta,[a,†,*] Malou van Zanten,[b,†] Mark McEwen,[a] Lynne Burrow,[a] Jack Beesley,[a] and Neil Piller[b]
[a]South Australian Biomedical Engineering at Flinders Medical Centre, Flinders Biomedical Engineering Department, Research & Teaching, Bedford Park SA 5042, Australia
[b]Flinders University, Department of Surgery, Lymphoedema Research Unit, Level 3, Flinders Medical Centre, Bedford Park, SA 5042, Australia

Abstract. Lymphoedema is a chronic progressive condition often producing significant morbidity. An in-depth understanding of an individual's lymphatic architecture is valuable both in the understanding of underlying pathology and for targeting and tailoring treatment. Severe lower limb injuries resulting in extensive loss of soft tissue require transposition of a flap consisting of muscle and/or soft tissue to close the defect. These patients are at risk of lymphoedema and little is known about lymphatic regeneration within the flap. Indocyanine green (ICG), a water-soluble dye, has proven useful for the imaging of lymphatic vessels. When injected into superficial tissues it binds to plasma proteins in lymph. By exposing the dye to specific wavelengths of light, ICG fluoresces with near-infrared light. Skin is relatively transparent to ICG fluorescence, enabling the visualization and characterization of superficial lymphatic vessels. An ICG fluorescence lymphatic vessel imager was manufactured to excite ICG and visualize real-time fluorescence as it travels through the lymphatic vessels. Animal studies showed successful ICG excitation and detection using this imager. Clinically, the imager has assisted researchers to visualize otherwise hidden superficial lymphatic pathways in patients postflap surgery. Preliminary results suggest superficial lymphatic vessels do not redevelop in muscle flaps. [DOI: 10.1117/1.JBO.20.6.066003]

Keywords: excitation; fluorescence; wavelength; lasers; lymphoedema; lymphatics; indocyanine green.

Paper 150108R received Mar. 2, 2015; accepted for publication May 12, 2015; published online Jun. 9, 2015.

1 Introduction

The lymphatic system has important immune system functions ensuring a healthy body homeostasis. Stasis in lymph drainage can occur as a result of congenital abnormality of the lymphatic system or as a result of secondary effects of cancer treatment. When lymph stasis is long term it will result in visible swelling, often called "chronic edema," but more accurately called "lymphoedema" due to the failed lymphatic drainage of the affected area in the body. Lymphoedema is a chronic progressive condition where the lymphatic fluid load exceeds the lymphatic system's transport capacity.[1] If left untreated, the fluid is replaced by fatty tissues and eventually by fibrotic tissues, both of which further compromise lymphatic function.

Posttrauma lymphatic response to a high-energy impact injury is both highly variable between patients and poorly understood as a whole. While edema, as a response to lower limb trauma, is a recognized phenomenon, it is not well understood when there is significant soft tissue injury accompanied by underlying bone injury. Methods of soft-tissue reconstruction in this patient group involve the introduction of vascularized tissue to promote wound healing and redevelopment of the skin–environment interface. This tissue includes variable amounts of muscle, epifascial fat and skin with an existing, well-developed vasculature. While the revascularization of the reconstructed area has been researched extensively, few studies have been conducted that target the understanding of the repair of lymphatic function.[2]

Discovery of lymphatic vessels as "white veins" is dated back to the early 15th century, but the lymphatic system has remained a mystery for quite some time.[3,4] Due to their thin delicate walls, their tendency to contract when touched, and their translucent appearance it has been challenging to visualize the lymphatic system in humans. In the 1950s, direct injection of a radio-opaque contrast agent allowed lymph vessel imaging to occur clinically.[4] Imaging, however, has remained invasive, often with mild radioactive contrast agents. In addition, current methods display poor spatial resolution as well as being costly and time consuming for both the patient undergoing the imaging and the radiologist.[5]

Indocyanine green (ICG) is a water-soluble tricarbocyanine dye. In the mid-1950s, ICG was introduced into diagnostic medicine for cardiac output measures, liver functioning, and ophthalmic angiography.[6] More recently (2005), its use for detecting sentinel lymph nodes in breast cancer patients was successfully explored by Kitai et al.[7] in Japan. Since then ICG has rapidly developed into a lymphatic imaging technique.

Due to its rapid binding to protein, high sensitive fluorescence properties, and low toxicity, ICG provides a minimally invasive method of lymph imaging. For superficial lymphatic imaging, ICG is injected into the intradermal layer temporarily creating high pressure in the interstitial space. It binds to protein

*Address all correspondence to: Olivia J. Pallotta, E-mail: olivia.pallotta@health.sa.gov.au

†These authors contributed equally to this work.

1083-3668/2015/$25.00 © 2015 SPIE

LYMPHATIC RESEARCH AND BIOLOGY
Volume 00, Number 00, 2015

DOI: 10.1089/lrb.2015.0026

Original Article

Inter-Changeability of Impedance Devices for Lymphedema Assessment

Malou van Zanten, BHth,[1] Neil Piller, BSc (Hons), PhD, FACP,[1] and Leigh C. Ward, BSc (Hons), PhD, RNUTR[2]

Abstract

Impedance technology is a popular technique for the early detection of lymphedema. The preferred approach is to use bioimpedance spectroscopy (BIS), with measurements being made with the subject lying supine, although attempts have been made to use single or multiple frequency impedance measurements obtained while the subject is standing. The aim of the present study was to determine the equivalence of these different approaches. Impedance measurements of the individual limbs of 37 healthy individuals were determined using both a stand-on, multi-frequency impedance device and a supine impedance spectroscopy instrument. Significant differences were found between the instruments in both absolute impedance values and, importantly, inter-limb impedance ratios. Since impedance ratios in healthy individuals provide the reference standard for detection of lymphedema, these data indicate that the methods are not interchangeable. Consideration of the errors associated with each method indicates that the BIS remains the preferred method for lymphedema detection.

Introduction

BIOELECTRICAL IMPEDANCE ANALYSIS (BIA) has become the method of choice for the early detection of lymphedema.[1,2] In BIA, the opposition, impedance, to the flow of an harmless electrical current through the body is measured. The measured impedance, denoted by convention Z and with the units ohm, is related to the volume of conductive fluid, total body water (TBW), within the body according to the following relationship:

$$TBW = \rho \frac{L^2}{Z} \quad \text{(Eq.1)}$$

where ρ is the specific resistivity of body fluids (ohm.cm), L is the distance between electrodes and Z is the measured impedance or resistance (ohm). Consequently, a change in the magnitude of Z is inversely proportional change in the volume of TBW. Impedance of body's tissues is dependent upon the frequency (Hz) of the applied electric current such that at low frequency, ideally zero, the current cannot pass across cell membranes that act as biological electrical capacitors and, hence, the change in impedance is now inversely proportional to the change in extracellular water volume:

$$ECW = \rho \frac{L^2}{Z_{low}} \quad \text{(Eq.2)}$$

where Z_{low} is the impedance measured at a low frequency. Impedance is a vector quantity comprising two components: reactance (Xc), the opposition to current flow due to the capacitive nature of cell membranes and resistance (R), the opposition to current flow due to the inherent resistance of tissue fluids such that

$$Z^2 = R^2 + Xc^2 \quad \text{(Eq.3)}$$

Equation 2 can thus be re-written as:

$$ECW = \rho \frac{L^2}{R_{low}} \quad \text{(Eq.4)}$$

where R_{low} is the resistance of the extracellular fluid. Lymph is an extracellular fluid. Lymphedema, a condition in which lymph fluid accumulates in tissues, elicits an increase in ECW that is measured as a decrease in R_{low} according to Equation 4.[3]

Owing to uncertainty over the precise value of specific resistivity, ρ, the presence of lymphedema is quantified as a change in R_{low} in an affected body region (e.g., arm or leg),

[1]Lymphoedema Research Unit, Department of Surgery, School of Medicine, Flinders University, South Australia, Australia.
[2]School Chemistry and Molecular Biosciences, The University of Queensland, Queensland, Australia.

Plastic and Reconstructive Surgery

The lymphatic response to injury with soft tissue reconstruction in high-energy open tibial fractures of the lower extremity

--Manuscript Draft--

Manuscript Number:	PRS-D-16-00241
Article Type:	Original Article
Full Title:	The lymphatic response to injury with soft tissue reconstruction in high-energy open tibial fractures of the lower extremity
Corresponding Author:	Malou Catharina van Zanten, B Health Flinders University of South Australia Adelaide, South Australia AUSTRALIA
Order of Authors:	Malou Catharina van Zanten, B Health
	Raakhi Mohanbhai Mistry, MD
	Hiroo Suami, MD, PhD
	Andrew Campbell-Lloyd, MD, FRACS
	James Peter Finkemeyer, MD
	Neil Brenton Piller, PhD
	Yugesh Caplash, MD, FRACS
Abstract:	Severe compound tibial fractures are associated with extensive soft tissue damage, resulting in disruption of lymphatic pathways that leave the patient at risk of developing chronic lymphoedema. There is limited data on lymphatic response following lower limb trauma. Indocyanine Green (ICG) fluorescence lymphography is a novel, real-time imaging technique for superficial lymphatic mapping. We planned to use this technique to image the superficial lymphatic vessels of the lower limbs in patients with severe compound tibial fracture. Hereby we investigated any changes to the normal anatomy of the lymphatic system. Baseline demographics, clinical and operative details were recorded in a prospective cohort of 17 patients who had undergone bone and soft tissue reconstruction following severe compound tibial fracture between 2009 and 2014. Normal lymphatic images were obtained from the patients' non-injured limbs as control. Of the 17 patients, 9 had free muscle flaps with split thickness skin graft, 1 full thickness skin graft, 6 local fasciocutaneous flaps and 1 pedicled gastrocnemius flap. None of the free muscle flaps demonstrated any functional lymphatic vessels; the fascio-cutaneous flaps and the skin graft demonstrated impaired lymphatic vessel function and dermal backflow pattern similar to that in lymphoedema. Local flaps demonstrated lymphatic blockage at the scar edge. Generally the uptake of the ICG was delayed in the affected leg compared to the control, which suggests a reduced lymph transport capacity. Severe compound fractures and the associated soft tissue injury can result in significant lymphatic disruption and an increased risk for the development of chronic lymphoedema.
Keywords:	Lymphatics; Indocyanine Green; lymphography; Reconstruction
Manuscript Classifications:	Flaps; Lower extremity acute trauma; lymphatic physiology and surgical approaches; Reconstructive--Lower Extremities
Additional Information:	
Question	Response