QMB Abstracts (Lymphatics in Health and Disease)

L1: Dissecting the Transcriptional Control of Lymphatic Endothelial Cell Identity

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The assembly of transcriptional machinery at transcriptional enhancer elements is responsible for orchestrating the precise temporal and spatial control of gene expression that is crucial for programming cell identity during development. Here, we describe a novel enhancer element important for regulating *Prox1* expression in lymphatic endothelial cells. This evolutionarily conserved enhancer is bound by key lymphatic transcriptional regulators including GATA2, FOXC2, NFATC1 and PROX1. Genome editing of the enhancer to remove 5 nucleotides encompassing the GATA2 binding site resulted in perinatal death of homozygous mutant mice due to profound lymphatic vascular defects. Lymphatic endothelial cells in enhancer mutant mice exhibited reduced expression of genes characteristic of lymphatic endothelial cell identity and elevated expression of genes characteristic of hemogenic endothelium and acquired the capacity to generate hematopoietic cells. These data not only reveal the first transcriptional enhancer element important for regulating *Prox1* expression and lymphatic endothelial cell identity, but also that lymphatic endothelium has hemogenic capacity, ordinarily repressed by *Prox1*.

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2. Kikin, O., L. D'Antonio and P.S. Bagga (2006). *QGRS Mapper: a web-based server for predicting G-quadruplexes in nucleotide sequences.* Nucleic acids research. 34: W676-82.

L2: IGF Signalling Regulates Cartilage-Lymphatic Guidance

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During development, migrating lymphatic vessels are guided by different tissues; blood vessels, mural cells and neurons have all been implicated in lymphatic vessel guidance. Lymphatic vessels are also known to develop adjacent to the outer perichondrial/periosteal layers of cartilage and bone, but the mechanisms that drive cartilage-lymphatic guidance are unknown.

The medial facial lymphatic (MFL) in the zebrafish head develops along the caudal surface of the hyoid cartilage, a subtype of craniofacial cartilage. Chondrocytes excrete collagen II, which is known to support lymphatic growth, but a collagen secretion mutant (*sec24d*) displayed normal MFL development. By contrast, a *kat6a* mutant unable to form hyoid cartilage had no MFL growth, while both laser and nitroreductase-mediated cartilage ablation further confirmed that the hyoid cartilage is required for MFL development.

After confirming that *kat6a* was not required cell autonomously for lymphatic growth, we next performed an RNA-Seq comparison between WT and *kat6a* mutant chondrocytes. We found that *pappa2*, a secreted metalloproteinase involved in activating Insulin-like growth factor (IGF) signalling, is downregulated in *kat6a* cartilage, and we further show that both *pappa2* morphants and crispants display MFL defects. To test the role of IGF signalling in lymphatic development, we used genetic and pharmacological approaches to inhibit or enhance this pathway.

Wildtype larvae treated with an IGFR inhibitor or overexpressing an IGF-binding protein (Igfbp5b) displayed reduced MFL growth. In contrast, pharmacological enhancement of IGF-signalling, overexpression of Igf2 or injection of recombinant human IGF2 were all able to partially rescue the MFL defect in *kat6a* mutants. Together this data shows that IGF signalling regulates the cartilage-lymphatic interaction required for MFL development and is the first study to link IGF signalling with embryonic lymphatic vessel development.

L3: The Molecular Basis for Lymphoedema and Surgical and Future Treatments - Part 1

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Introduction: Lymphoedema, a devastating pathological interstitial fluid accumulation causing significant functional impairment, infection and reduced quality-of-life, results from developmental derangement or mechanical lymphatic disturbance. Whilst physical therapy and compression remain mainstays of treatment, surgery has been shown to be potentially beneficial; however, selection protocols for long-term objective outcomes measures remain variable. We review our series of nearly 700 cases treated surgically (including 500 LVAs) and discuss innovations enabling improved patient selection, techniques, and outcomes.

Methods: We compare static/dynamic compression therapy vs. surgery (LVA, free lymph node transfer, liposuction or debulking, or combinations) using standardised quantitation. A lymphatic subtype image-based algorithmic approach was designed to select patients for non-surgical vs. surgical treatment. Observations in the first 100 cases were applied to the following 100. Following the application of novel improvements in surgical techniques, follow-up was performed for one year, using independent therapist review, quantitative questionnaires combining objective and subjective measures and lymphangiography.

Results: Patients selected for surgery underwent assessment of outcome by intervention and stage. 853 (30%) patients were deemed candidates (503 LVA, 48 free LN transfer, 146 liposuction, 113 surgical debulking, 43 patients did not progress to surgery). 43/853 (5%) of surgical candidates improved with 3-months optimal dynamic therapy, no longer requiring surgery (dynamic was superior to static compression in limb volume reduction). Surgical outcomes were quantified by lymphoedema stage and cause. Tissue excision or debulking were applied palliatively in inoperable/failed surgical candidates, and provided quality-of-life benefit.

Conclusions: Lymphatics microsurgery is not new, however, as in any surgical technique, patient selection and application, as well as ongoing technical refinement is critical to success. In appropriately selected lymphoedema patients, there is a role for the right surgical method. We present findings and modifications to selection and non-operative and operative treatment, emphasising physiological and anatomical variations to improve LVA surgery.

L4: The Molecular Basis for Lymphoedema and Surgical and Future Treatments - Part 2

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Introduction: The causes of secondary lymphoedema included treatments of lymph node basins for cancer (surgery and radiotherapy), trauma, or parasitic obstruction, with a combination of surgery and radiotherapy are significantly additive. Amongst known molecular pathways regulating the lymphatic system, the VEGF-C/D-VEGFR3 axis is the most well-known. Nevertheless, molecular mechanisms underlying damage to lymphatic vessels, and particularly the lymphatic endothelial cells (LEC) that constitute them, by these treatment modalities, remain poorly understood. Furthermore, most lymphoedema patients remain untreatable. Therefore, a stronger mechanistic understanding, improved surgical selection and techniques, and potential molecular therapies for surgically untreatable patients are urgently required. Here we explore mechanistic elements that impair lymphatic regeneration and promote lymphoedema.

Methods: To explore the reasons why radiation can cause lymphoedema and render many patients untreatable, we used a combination of cell-based assays, biochemistry and animal models of lymphatic injury to examine the molecular mechanisms behind LEC injury and subsequent effects on lymphatic vessels, particularly the role of the VEGF-C/VEGF-D/VEGFR-3 lymphangiogenic signalling pathway, in lymphatic injury underpinning the development of lymphoedema.

Results: Mechanistically, we demonstrate that radiotherapy selectively impairs key LEC functions needed for new lymphatic vessel growth (lymphangiogenesis). This effect is mediated by attenuation of VEGFR-3 signalling and downstream signalling cascades. VEGFR-3 protein levels were downregulated in LEC that were exposed to radiation, and LEC were therefore selectively less responsive to VEGF-C and VEGF-D. These findings were validated in our animal models of radiation and surgical injury.

Conclusions: In order to understand the deleterious effects of radiation therapy and surgery, we examined the molecular changes that may lead to making many patients unsuitable for surgery. Our data provide mechanistic insight into injury sustained by LEC and lymphatics during surgical and radiotherapy cancer treatments and underscore the importance of research in guiding the applications of all future lymphoedema therapies.

L5: Anatomic Changes of the Lymphatic System after Lymph Node Dissection

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Background: Lymphoedema is a debilitating condition represented by swelling in the affected body regions after cancer treatment. Lymphoedema is caused by reduced lymph fluid transport capacity mainly after lymph node dissection. The current unsolved issue in lymphoedema is that we cannot predict who will be affected. The gross anatomic study of the lymphatic system is one of the promising approaches to shed light on the pathophysiology of lymphoedema. Clinical imaging studies including indocyanine green (ICG) fluorescent lymphography have reported post-operative anatomical changes in the lymphatic system.

Materials and Methods: The anatomy of the lymphatic system in the upper and lower limbs was investigated by using fresh human cadaver specimens. The presenter's original method was applied to visualise the lymph collectors. The lymph collectors were traced until they connected to their corresponding nodes and the relationships between the lymph collectors and nodes were investigated. The animal experiments were conducted using mongrel dogs. Lymph nodes in the cervical and axillary regions were dissected and the operated area was irradiated. Lymphatic imaging including ICG lymphography and lymphangiography was applied in the forelimb and changes in the lymphatic structures were analysed. Clinical studies with ICG lymphography were performed in patients with cancer-related lymphoedema who visited the ALERT clinic. ALERT ICG protocols were applied to the patients and lymphatic drainage regions were recorded.

Results: The human cadaver studies provided baseline information in normal lymphatic anatomy. The dog experiment demonstrated that collateral lymphatic pathways were developed and connected to the remaining lymph node for maintaining lymph drainage from the affected forelimb. ICG lymphography in patients with lymphoedema demonstrated distinct patterns of drainage regions.

Conclusions: The findings through these studies suggested that the lymphatic system may have a homing mechanism, which allows the severed lymphatic vessels to detect and connect to adjacent remaining lymph nodes.

1. Suami H. (2020) *Anatomical Theories of the Pathophysiology of Cancer-Related Lymphoedema.* Cancers (Basel). 23;12:1338.
2. Suami H, Scaglioni MF, Dixon KA, Tailor RC. (2016) *Interaction between vascularized lymph node transfer and recipient lymphatics after lymph node dissection-a pilot study in a canine model.* J Surg Res. 204(2):418-427.

L6: Modern Studies in the Anatomy and Physiology of the Thoracic Duct, Lymphovenous Junction, and Cisterna Chyli

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The lymphovenous junction (LVJ) of the thoracic duct (TD) is the principle outlet of the lymphatic system. Interest in this junction is growing as its role in lymphatic outflow obstruction is being realized, and as minimally invasive procedures for accessing the terminal TD become more common. Despite the growing clinical significance of the TD and LVJ, its precise form and function remain unclear. A systematic review of the literature surrounding the TD, LVJ, and the cisterna chyli will be discussed. Original cadaveric studies of the TD and LVJ will be presented along with subsequent dynamic ultrasound investigations of the region. The body of work represents an ongoing effort to characterise the anatomy and physiology of a region of the human body that in many ways remains 'terra incognita'.

1. Moazzam S, O'Hagan LA, Itkin M, Clarke A, Phillips ARJ, Windsor JA, Mirjalili SA. *The cisterna chyli: a systematic review of definition, prevalence and anatomy*. Am J Physiol Heart Circ. 2022;323(5):H1010-H1018.
2. Hinton LR, O'Hagan LA, Griffiths AP, Phillips ARJ, Windsor JA, Mirjalili SA. *The effect of respiration and body position on terminal thoracic duct diameter and the lymphovenous junction: An exploratory ultrasound study.* Clin Anat. 2022;35(4):447-453.
3. O'Hagan LA, Windsor JA, Itkin M, Russell PS, Phillips ARJ, Mirjalili SA. *The lymphovenous junction of the thoracic duct: a systematic review of its structural and functional anatomy.* Lymphat Res Biol. 2021;19(3):215-222.
4. O'Hagan LA, Windsor JA, Phillips ARJ, Itkin M, Russell PS, Mirjalili SA. *Anatomy of the lymphovenous valve of the thoracic duct in humans*. J Anat. 2020;236(6):1146-1153.

L7: Magnetic Resonance Imaging to Understand Lymphatic Anatomy and Function in the Torso

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The anatomy of the lymphatic system has been shown to be highly variable, and so understanding this anatomy and its consequences on function in individuals and populations is critical to any medical intervention that targets this system. Fluid that passes through the capillaries is transferred to the lymph network and returned to the circulation via the thoracic duct. Near to the thoracic duct the flow of lymph is influenced by both respiratory pressures the nature of blood flow within the systemic circulation. The nature of this interaction between respiratory, circulatory and lymph systems is not well understood.

Here we present a magnetic resonance imaging approach to understanding the anatomy and function of the major lymphatic vessels in the torso. All scanning was conducted on a 3T GE Medical Systems scanner at Mātai Medical Research Institute (Gisborne, New Zealand). Heavily weighted T2 imaging was conducted to highlight the slow moving fluid within the major draining lymphatic vessels within the torso, along with their relationship with the venous circulation. We will discuss methodologies to segment key anatomic features from this imaging, alongside preliminary biomechanical simulation studies that provide a link between anatomical structure of the lymph vessels and their function in an individual. Finally, we discuss strategies for quantifying flow in the lymphatic vessels using magnetic resonance imaging that can be used to gain further insights into the function of the lymphatic vessels non-invasively.

L8: Computational Modelling of the Lymphatic System using a Multiphysics Approach

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Computational modelling of the lymphatic system offers a powerful tool to simulate its function during health and disease. Historically, significant challenges have emerged when developing models of the lymphatics largely due to a lack of imaging and experimental data[1]. Furthermore, currently there are only two published studies that have simulated lymph flow through an entire human lymphatic network[2,3]. However, neither of these studies used human imaging data for accurate model parameterisation, and no model has simulated flow through complete networks of lymphatic vessels and lymph nodes.

To address this, we are developing a comprehensive computational model to simulate lymph flow dynamics through each functional component of the lymphatic system. We have developed a bond graph model of lymph flow through the initial and pre-collecting lymphatic vessels. We will couple this with an existing one-dimensional model of collecting vessels[4] and develop a novel bond graph model of lymph flow through lymph nodes. The bond graph approach provides several advantages over standard modelling approaches by conserving physical quantities such as mass and momentum during simulations. In addition, the one-dimensional collecting vessel model simulates smooth muscle contractions of the vessel walls, based on a modified FitzHugh-Nagumo model for action potentials, and wave propagation.

We will present our initial modelling results and describe the future roadmap to enable *in silico* modelling of each component of the human lymphatic system. Our goal is to simulate lymph flow dynamics in health and in diseases such as lymphoedema. In future, we aim to couple this with models of the cardiovascular system and use it as a framework to simulate other scenarios such as cancer metastasis, drug delivery, and critical illness.

1. Moore, J. E. & Bertram, C. D. *Lymphatic System Flows*. Annu Rev Fluid Mech 50, 459–482 (2018).
2. Reddy, N., Krouskop, T. & Newell, P. *A Computer Model of the Lymphatic System*. Comput. Biol. Med. 7, 181–197 (1977).
3. Savinkov, R. *et al. Graph theory for modeling and analysis of the human lymphatic system*. Mathematics 8, 1–18 (2020).
4. Contarino, C. & Toro, E. F. *A one-dimensional mathematical model of collecting lymphatics coupled with an electro-fluid-mechanical contraction model and valve dynamics*. Biomech Model Mechanobiol 17, 1687–1714 (2018).

L9: Central Nervous System Draining Lymphatics in Health, Aging, and Disease: Mechanisms for their Decline and Therapeutic Interventions

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Despite the established dogma of central nervous system (CNS) immune privilege, neuroimmune interactions play an active role in diverse neurological disorders. Of particular importance, the recent identification of a lymphatic network situated in the meninges—border tissues surrounding the brain—have illuminated a site for surveillance of central nervous antigens and the clearance of brain waste. Whilst functional in steady-state conditions, this lymphatic network becomes dysfunctional during aging, and in diverse neurological disorders, leading to the build-up of pathological waste proteins in the brain, including amyloid beta in Alzheimer's disease and alpha synuclein in Parkinson's disease. However, the mechanisms driving this decline are unknown, making therapeutic intervention strategies elusive. Here, I will discuss emerging evidence for diverse mechanisms that drive meningeal lymphatic dysfunction in aging and disease, with relevance for therapeutic manipulation.

L10: Understanding Pulmonary Lymphatic Vessels: A Multi-Modal Research Project of their Anatomy, Function, and Pathophysiological Impact

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Despite significant advances in lymphatic research, the human pulmonary lymphatic vessels (PLVs) remain notably understudied. The importance of PLVs in human physiology in health and disease cannot be overstated, but our understanding of their intricate layout and operations, particularly regarding intrapulmonary and extrapulmonary drainage paths, is far from complete. The anatomical arrangement of these drainage paths within PLVs continues to be a significant challenge for scientists. We have embarked on a thorough exploration of PLVS, from literature searches of existing anatomical descriptions of PLV to computer simulation models of their behaviours, in an attempt to bridge this knowledge gap. This overarching project brings together various scientific studies and research findings on the subject, providing a comprehensive understanding of PLVs. This method has aided in identifying key areas where our understanding is lacking and suggests avenues for future research.

We focused on cadaveric samples in particular, which will allow us to produce a comprehensive description of PLVs in humans using traditional histological techniques and modern immunohistochemistry markers. The former will give us a high-resolution overview of the microanatomy of PLVs in various segments of the lung, whereas the latter will allow us to identify the structural arrangement of PLVs. In addition to this approach, we are using MRI imaging to identify extrapulmonary lymphatic drainage paths and combining our findings in a PLV in silico model.

As a result, we will be able to provide the first comprehensive description of PLV anatomical arrangement in humans and their behaviour in various pathological conditions. This comprehensive study, which included a literature review, direct observation, and advanced imaging techniques, represents a significant advancement in our understanding of the human pulmonary lymphatic system and serves as a foundation for future research aimed at comprehensively understanding this critical component of pulmonary physiology.

L11: A Biophysically-Based *in silico* Model of Pulmonary Lymphatic Function

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Despite the lymphatics' vital roles in fluid homeostasis and immune functioning, there are huge gaps in our knowledge of how they work in health and disease. There is growing evidence that lymphatic dysfunction is involved in various pathologies. For example lung lymphatic dysfunction has recently been implicated in lung injury in humans after cigarette smoke exposure and COVID-19. This 'forgotten' circulation is rarely considered in studies of lung dysfunction and is difficult to study experimentally. We have developed an anatomically structured *in silico* model of pulmonary lymphatic function[1] to gain insights of this system in health and disease.

The model uses biophysical principles to estimate the transfer of fluid between the capillary, interstitium, lymphatic vessels, and 'overflow' into the alveolar space. The lymphatic model is coupled with models for lung perfusion and ventilation to introduce physiologically driven changes in blood and tissue pressures. To validate the model, we compared predicted values with experimental values from the literature under conditions of increased vascular pressures and endothelial permeability.

The model returned physiologically reasonable values for all applications, predicting pulmonary oedema when left atrial pressure reached 25 mmHg and with increased permeability. The model provides physiologically reasonable predictions of whole lung lymphatic clearance, and insight into the microstructural interactions that influence this. The filtration coefficient, pressures, and interstitial capacity were important factors governing lymph flux.

This model presents a novel approach to understanding the interaction between cardiopulmonary dysfunction and pulmonary lymphatic function, using anatomically structured models and biophysical equations to estimate fluid transport from blood to interstitial and lymphatic flux. We plan to use this model to investigate intersubject and pathophysiological changes.

1. Ashworth, E., Burrowes, K., Clark, A., Ebrahimi, B.S., Tawhai, M (2023). *An in silico approach to understanding the interaction between cardiovascular and pulmonary lymphatic dysfunction*. AJP-Heart and Circulatory Physiology. 2023;324(3):H318-29.

L12: Cancer Rehabilitation in the Head and Neck Population - Observations Over a 12-Month Period

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Head and neck cancer rehabilitation is considered one of the more complex populations to rehabilitate due to the breadth of issues that can arise, and the significant impact this has on basic activities of daily living[1]. These issues present following surgical and radiotherapy interventions, and include high rates of depression and anxiety, reduced movement, weakness, and pain in the shoulder/jaw/neck, changes to speech and swallowing (which impacts nutritional intake) and lymphoedema.

As part of lymphoedema physiotherapy clinical input, outcome measurements are used to identify and prioritise each client's needs. With the aim of improving the care that is provided to this client population, this observational clinical data was analysed retrospectively to establish what trends occur at 3, 6, and 12 months following surgical intervention; with comparisons made between the radiotherapy and non-radiotherapy clients.

Outcome measures that have been included in this comparison are active glenohumeral flexion and abduction, mouth opening, neck rotation, shoulder strength using a dynamometer, and lymphoedema outcome measurements via neck circumference and Tissue Water Constant using the Delfin Lymphscanner.

The results identify that clients whom underwent radiotherapy achieved smaller gains at all time points in shoulder flexion, abduction, and mouth opening, compared to those that did not require radiotherapy. The exception to this being mouth opening, whereby, at 12-months there is no difference between groups. Neck rotation, shoulder dynamometry and Lymphscanner outcome measurements improved in individuals that did not require radiotherapy, however for those that received radiotherapy, a significant deterioration is noted at 6-months following surgery, and a small deterioration is noted in neck circumference in this group.

1. Ringash, J., Bernstein, L., Devins, G., Dunphy, C., Giuliani, M., Martino, R., & McEwen, S. (2018). *Head and Neck Cancer Survivorship: Learning the Needs, Meeting the Needs*. Seminars in Radiation Oncology, 28(1), 64-74

L13: Re-Configuring Life: Living with Moderate to Severe Lower Limb Lymphoedema in Aotearoa NZ

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Background: This doctoral study is the first known qualitative research focusing on how people adapt to living with moderate to severe lower limb lymphoedema in Aotearoa NZ. The study explicates a process of reconfiguring a new normal in response to living with long term swelling.

Methods: Qualitative research, with a grounded theory approach, was used to analyse data from 12 adults living with moderate to severe lower limb lymphoedema, which generated a theory.

Findings: The onset of lymphoedema is characterised by disruptive, intrusive, physical symptoms that destabilise normality, impact mobility, appearance, sense of self, and provoke wardrobe dilemmas. Livelihoods were disrupted, particularly for men in physical occupations. Self-care routines were demoralisingly demanding. Physical symptoms provoked feelings of distress and despair, which often created a drive to pursue solutions. A turning point was reached when hope replaced despair. The lymphoedema therapist was often key to activating change, through supporting autonomy and a non-judgemental approach. Participants reconfigured a new normal through what they learnt, what they did, and through how they came to be. Participants acquiesced to lymphoedema as a greater force which could be managed but not cured.

Discussion: The theory of reconfiguring includes a step by step process explaining how participants regained control through becoming amenable to change with the support of the lymphoedema therapist. Self-care is not care done alone.

L14: Lymphatic Malfunctions in Atherosclerosis and Secondary Lymphedema (Keynote Address)

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The ever-growing research on lymphatic biology has clearly identified lymphatic vessels as key players in maintaining our body healthy through their functional roles in tissue fluid homeostasis, immunosurveillance, lipid metabolism and inflammation. During the first part of my talk, I would present our work showing that hypercholesterolemia associated with atherosclerosis leads to lymphatic malfunctions which in turn contribute to the progression of the disease. In the second part of my presentation, I would share our recent findings on human secondary lymphedema pathogenesis revealing that lymphatic drainage insufficiency in this condition not only results in fluid but also lipid accumulation, especially cholesterol. In response to this excessive deposition of cholesterol, adipocytes become hypertrophic, and at saturation point, they eventually die leading to adipose tissue loss and inflammation. At this more advanced stage of secondary lymphedema, fibrosis prevails, and macrophage and fibroblast transform into foams cells. Based on these clinical data, we propose that targeting lipids in secondary lymphedema may be a promising strategy to prevent its progression.

L15: Portable 3D Fluorescence Lymphography for Clinical Applications

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Lymphoedema is a disease where a dysfunctional lymphatic system results in chronic swelling of a body region. Management is currently the best option for preventing disease progression and improving patient outcomes. Current clinical fluorescence lymphography tools, used to map the underlying lymphatic dysfunction, are limited as they do not enable the creation of 3D maps of lymphatics throughout affected limbs. In this work we present recent results from a new portable and low-cost clinical imaging system for creating 3D maps of lymphatics.

The custom 3D imaging system comprises three 20-megapixel cameras and illumination hardware to provide both visible light for skin surface texture and 734 nm excitation light for fluorescence of indocyanine green (ICG). The visible skin texture of limbs can be imaged simultaneously with the near-infrared fluorescence from ICG in the underlying lymphatics. We performed a clinical pilot study and imaged participants with upper limb and lower limb lymphoedema following a clinical ICG lymphography imaging protocol[1]. The imaging system was able to provide detailed 3D models of limbs from a single view with colourmaps of underlying ICG distribution, enabling identification of lymphatic dysfunction including regions of dermal backflow. This new 3D fluorescence lymphography imaging system is promising for clinical application due to its portability and low cost.

1. Suami, H. *et al. A new indocyanine green fluorescence lymphography protocol for diagnostic assessment of lower limb lymphoedema*. Journal of Plastic, Reconstructive and Aesthetic Surgery **75**, 3946–55 (2022).

L16: Functionalised Peptides Show Absorption in the Stomach and Small Intestine and Potential Lymphatic Uptake

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Introduction: The development of orally administered peptide therapeutics has been hindered by poor oral bioavailability, necessitating administration via injection. Understanding the sites and mechanisms of absorption of peptides from the gastrointestinal tract is essential for designing more effective delivery systems. This study examines the absorption sites and lymphatic uptake of two functionalised peptides with varying molecular weight and functionalisation: PTG-01 (2794 Da functionalised with fatty acid) and PTG-02 (1845 Da functionalised with small-molecule albumin-binding moiety).

Methods: Peptides were administered to anaesthetised rats into the small intestine or stomach with/out pylorus ligation and serum samples collected over time to identify the primary absorption site: stomach or intestine. The absorption route was examined by comparing lymph and serum pharmacokinetics after intestinal delivery of the peptides in mesenteric- or thoracic lymph-duct cannulated rats. The effect of co-administration with oleic acid on absorption and lymphatic uptake was also assessed. Serum and lymph concentrations of the peptides were quantified using a validated LC-MS/MS method.

Results: After oral administration, PTG-01 serum concentrations were lower in rats with stomach ligation compared to stomach-intact, suggesting small intestine absorption. Conversely, PTG-02 serum concentrations were higher with stomach ligation compared to stomach-intact, suggesting stomach absorption. PTG-02 serum concentrations also increased after dosing in the small intestine versus stomach with/out ligation, indicating absorption in both stomach and intestine. Lymphatic uptake of PTG-02 and PTG-01 was relatively low, but higher peptide concentrations were observed in lymph compared to serum for some rats and
time points, particularly for PTG-02, suggesting potential direct lymph uptake from the intestine. Co-administration with oleic acid enhanced PTG-01 absorption into blood and lymph, suggesting a strategy for improved oral bioavailability.

Conclusion: PTG-01 was primarily absorbed in the intestine, while PTG-02 was absorbed in both the stomach and small intestine. Additionally, there is evidence of lymphatic uptake, potentially due to association with albumin. Future studies will investigate absorption mechanisms, optimal conjugations and formulations for enhanced absorption and lymphatic uptake, and increased delivery to target sites in lymphatics, such as immune cells.

L17: A Lipid-Based Formulation Facilitates Lymphatic Uptake of a Protease Inhibitor

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Dabigatran etexilate (DABE) is a lipophilic prodrug of dabigatran which is a protease inhibitor that inhibits both trypsin and thrombin and is used clinically as an anticoagulant. Recently, pancreatic protease such as trypsin in the gut-lymph have been shown to promote organ failure in acute and critical illness (ACI). Most drugs access the gut-lymph in low quantities as they are rapidly transported away from the intestine via the blood. Here, we aimed to deliver DABE directly to the gut-lymph to inactivate proteases and potentially offer a targeted therapeutic strategy for organ failure in ACI. A series of lipid-based formulations (LBF) were designed and tested in vitro for their potential to self-emulsify, solubilise DABE and remain stable over time by ensuring the LBF maintained self-emulsification properties and that DABE remained stable without undergoing hydrolysis. In vitro conversion studies were performed to assess the stability of DABE and its conversion to dabigatran under simulated gastrointestinal digestion conditions and in plasma/lymph samples. Ultimately, a Type III A LBF with high self-emulsifying ability of DABE was chosen for progression into in vivo studies in male Sprague Dawley rats to confirm the lymphatic uptake and plasma pharmacokinetics. DABE demonstrated rapid conversion to both an intermediate compound BIBR1087 and dabigatran, both in vitro and in vivo in plasma/lymph. The main species present in both plasma and lymph in vivo was dabigatran and mass transport in lymph of DABE and dabigatran was minimal (~0.5% of dose). Importantly, the concentration of DABE in lymph was substantially (20-176 fold) higher than in plasma supporting that if the prodrug were stable and did not rapidly convert to dabigatran it would be lymphatically transported. Future studies will investigate strategies to enhance the stability of the prodrug during absorption and optimise the formulation for improved lymphatic uptake.

L18: IGF Signalling Regulates Medial Facial Lymphatic Vessel Development in Zebrafish

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Lymphatic vessels are known to develop using non-lymphatic tissues, such as blood vessels and motoneurons, as guidance structures. Lymphatic development is also found adjacent to the outer perichondrial/periosteal layers of mammalian cartilage and bone, but the molecular mechanisms that drive cartilage-lymphatic guidance are unknown.

The medial facial lymphatic (MFL) in the zebrafish head develops along the caudal surface of the hyoid cartilage, a subtype of craniofacial cartilage. A *kat6a* mutant unable to form hyoid cartilage had no MFL growth, suggesting that the hyoid cartilage is required for MFL development. An RNA-seq comparison between WT and *kat6a* mutant chondrocytes revealed the downregulation of *pappa2*, a secreted metalloproteinase involved in activating Insulin-like growth factor (IGF) signalling, in *kat6a* cartilage. To test the role of IGF signalling in lymphatic development, we used genetic and pharmacological approaches to either inhibit or enhance this pathway.

Wildtype larvae treated with an IGFR inhibitor had a reduction in lymphatic vessel development throughout the larvae, including the MFL. In contrast, pharmacological enhancement of IGF-signalling specifically increased MFL growth in wild-type animals and partially rescued the MFL defect in *kat6a* mutants. Consistent with pharmacological experiments, overexpression of an IGF-binding protein (Igfbp5b) from chondrocytes led to MFL defects in wildtype larvae. In contrast, overexpression of Igf2 or injection of recombinant human IGF2 partially rescued the MFL defect in *kat6a* mutants.

Together this study shows that Igf-signalling is involved in cartilage-guided lymphangiogenesis and is the first study to link IGF signalling with embryonic lymphatic vessel development.

L19: Fat-Associated Lymphoid Clusters in Immune-Metabolic Diseases

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Visceral adipose tissue (VAT) encases mesenteric lymphatic vessels and lymph nodes through which lymph is transported from the intestine and mesentery. In obesity, the accumulation of VAT within the abdomen stimulates inflammatory and metabolic changes that promote insulin resistance. Live imaging showed compromised lymph vessels in obesity become 'leaky', providing an entry of inflammatory cells and altered lipid metabolites in intestinal lymph to VAT. We showed that 'obese lymph' promotes adipogenesis, lipolysis and adipocyte insulin resistance [1]. In parallel, VAT is also a reservoir of fat-associated lymphoid clusters (FALCs). FALCs form part of the mesenteric lymphatics and act as immune sentinels to recruit large numbers of inflammatory cells and support rapid B cell responses in serous cavities during infection [2, 3]. Imaging data reveal that FALCs proliferate significantly in obesity, likely contributing to B cell-driven inflammation in metabolic disease. More importantly, FALCs develop localised, ectopic lymph vessels in obesity and B cells are exclusively recruited to FALCs when lymph vessels are present. This suggests that B cells derived from lymph leakage to VAT are recruited to FALCs via the lymph vessels, and that FALCs facilitate B cell expansion and promote insulin resistance and inflammation. Preliminary flow cytometry analysis has shown heterogenous lymphatic endothelial cells (LECs) populations and macrophages in VAT, compared to subcutaneous adipose tissue and the intestinal lamina propria in obesity. These LECs and macrophages appeared to show distinct profiles in markers relating to immune activation and stem cell potential.

The data present a novel pathophysiological mechanism of mesenteric lymphatic dysfunction for the development of visceral obesity, inflammation and insulin resistance, and the identification of potential therapeutic targets to treat immune-metabolic disease.

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L20: Targeting Lymphoid Niches to Promote CD8+ T Cell Memory

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Activation and differentiation of T cells is an essential part of orchestrating the adaptive immune response. These processes determine precisely how responses are tailored towards the pathogen and if immune protection is established. $CD8^+$ T cells dynamically migrate to distinct lymphoid niches to enable interactions with cellular partners that determine their effector function and the formation of memory.

The protective capacity of TCF-1+ stem cell-like memory CD8+ T (T_{SCM}) cells lie in their long-term persistence, potent proliferative capacity and ability to generate effectors upon rechallenge. As such, T_{SCM} cells are key cellular targets for vaccination and immunotherapy, yet how this cell population can be therapeutically promoted is unclear. Here, we report that T_{SCM} generation is directed with early, limited inhibition of the type I interferon (IFN) receptor (IFNAR) during viral infection and vaccination. Using scRNAseq and multiparameter flow cytometry, we identified that T_{SCM} cells generated with IFNAR blocking were distinct from exhausted T cells and remained increased following viral clearance. Three-dimensional light sheet imaging of intact lymph nodes revealed elevated T_{SCM} cell differentiation correlated with T cell retention within the lymph node paracortex. Inhibition of IFNAR signaling drove a compensatory increase in IFNγ production, which increased CXCR3 chemokine abundance and disrupted gradient formation. In the absence of both IFNAR and IFNγ, chemokine expression was lost, which again ablated gradient formation to promote paracortex location and T_{SCM} cell fate. Applying these findings, we combined early short-term IFNAR inhibition with mRNA-LNP vaccination to specifically generate T_{SCM} cells and confer enhanced prophylactic protection against chronic infection. Our findings support the design of vaccine and adjuvant approaches that elicit T_{SCM} cells for protection against infectious pathogens or for therapeutic cancer vaccination.

L21: Comprehensive 3D Imaging and Computational Analysis of Blood and Lymphatic Networks Across Entire Lymph Nodes

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Long-lasting immune responses against infections and cancer begin within highly organised immune organs such as lymph nodes. A central feature of their specialised microanatomy is an intricate network of blood and lymphatic channels, which serve as entry and exit routes for immune cells and lymph, thereby orchestrating the course of immune cell migration and antigen transmission. However, we currently lack understanding of the global organisation of both vascular systems and their structural dynamism during inflammation, when lymph nodes increase rapidly in size in response to changes in cell trafficking. Using a unique automated confocal imaging system, we generated unprecedented 3D images of blood and lymphatic channels in murine lymph nodes at subcellular resolution, providing new insights into their labyrinthine arrangement and regional specialisation. To make these large datasets accessible for quantification, we developed custom image-processing and analysis tools and measured the network topology including its branching and gap morphology. In particular, we generated extensive 3D imagery of a fine lymphatic channel system referred to as the conduit system and identified the compartments it supplies deep within the lymph node. Computational quantification of the conduit topology across the central T cell zone revealed significant differences in conduit density between the deep and superficial T cell zones and provided us with a realistic ‘road map’ for simulating T cell motility. The latest advances in this 3D imaging technology now offer improved image resolution and speed, allowing us to investigate the structural dynamism of these channel systems globally while being able to zoom in at fine details at the single cell level. This novel imagery broadens our understanding of the highly organised networks within lymph nodes, and with the help of topological mapping this information can now be used to advance sophisticated models of lymph node biology.

L22: A Critical Role for Endothelium in the Early Pre-Metastatic Niche

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Metastasis, the spread of cancer cells from the primary tumour to distant organs, is responsible for >90% of cancer-related deaths. Lymphatic and blood vessels are essential for transporting tumour cells throughout the body, with extravasation into distant tissues representing a key rate-limiting step in metastasis. Importantly, primary tumours can pre-emptively manipulate distant organ microenvironments prior to the arrival of metastatic cells to promote their infiltration and survival by creating “pre-metastatic niches” (PreMN) through secretion of various circulating factors. Despite their position as the primary interface between circulation and tissue, the role of endothelial cells in the PreMN remains poorly characterised.

Here we have sought to elucidate the responses and roles of endothelial cells at the earliest phases of PreMN formation in clinically important organs using single-cell RNA sequencing (scRNA-seq). As pre-metastatic tissues are rarely biopsied from human patients, we modelled the evolution of the PreMN over time using specific derivatives of the 4T1 syngeneic orthotopic murine model of metastatic breast cancer. We focused on brain and lung, as frequent and highly morbid sites of metastasis, and the tumour-draining lymph node due to its prognostic importance in cancer staging. The timing of PreMN and metastasis development in each of these organs was established using known markers (e.g. myeloid cell infiltration, tumour markers) before earlier timepoints were selected for analysis by scRNA-seq. Our results showed that in each organ, subtypes of endothelial cells differ in their pre-metastatic responses. For example, in lungs at 12 days after tumour inoculation, one capillary type exhibited >250 differentially-expressed genes, whereas another exhibited only 5, suggesting that endothelial subtypes are selectively involved in PreMN formation. Our results highlight early and heterogeneous organ- and cell type-specific responses of endothelium during PreMN evolution and have the potential to identify novel prognostic markers and therapeutic targets to combat metastasis.

L23: The Gut-Lymph Model Provides a New Paradigm for the Treatment of Organ Failure

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Organ failure has been described as the plague of modern medicine. Today, advances in intensive care management allow most patients to survive their primary or admission disease (such as haemorrhagic shock, trauma, pneumonia). However, some patients survive long enough to succumb to catastrophic multiple organ failure (MOF), the secondary disease. This MOF is the leading cause of death in intensive care units around the world and represents a massive health and economic burden.

The treatment administered for MOF is sophisticated but does not amount to more than physiological support (e.g. ventilation to assist breathing, inotropes to assist perfusion, dialysis to assist waste removal). Without a clear understanding of the critical drivers of MOF there is no specific treatment and new thinking is urgently needed.

Two clinical observations over 25 years ago stimulated our research program to investigate lymphatic dysfunction in organ failure. The first observation was that the pattern of MOF is strikingly similar, irrespective of the primary disease, suggesting a common mechanism. The second observation is that whenever patients with MOF have their abdomens opened the gastrointestinal track looks sick and dusky and sometimes frankly ischaemic. This suggested that the gut may have a key role to play, and our review of the literature revealed a 'gut-lymph hypothesis' based on laboratory work by Deitch who never attempted to translate his findings to the clinic. We also discovered a hidden literature from behind the iron curtain that used external drainage of lymph to reduce the severity of a wide range of diseases. Since then we have provided additional experimental support for what is now an established 'gut-lymph model' of organ failure. This model states that gut injury in acute and critical illness results in compositional and toxic changes in lymph draining from the gut. This lymph bypasses the liver and enters the systemic circulation immediately upstream of the organs that fail most often, the heart, lungs and kidneys. Two of our important earlier studies that support this gut-lymph model will be highlighted along with an overview of our current program. The model has provided a new paradigm that has led to a number of translatable findings, and some of these will be discussed in more detail by my colleagues at this meeting.

L24: Gene Expression Changes in the Lungs by Critical Illness and the Impact by the Mesenteric Lymph Drainage

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Critical illness (CI) is a major global health burden and the leading cause of death in the intensive care units. It can originate from a wide range of aetiologies and often progresses through a common pathway of increasing severity – systemic inflammatory response syndrome and multiple organ dysfunction syndrome (MODS). Although the exact pathophysiological mechanism is not fully understood, mesenteric lymph (ML) has been recognised as an important channel that carries toxic mediators in MODS.

To better understand how ML damages the lungs (the key end organs most frequently fail in MODS), we investigated the early gene expression changes in lungs of rat CI models (acute pancreatitis, sepsis and gut ischemia-reperfusion injury) and their respective Sham controls, with and without ML drainage, by rat Clariom D arrays. We found significant gene expression changes in the lungs following CI in all three models, and the ML drainage altered the gene expression profile of those diseased lungs. To support the results, we further performed qRT-PCR on the lung tissues, proteome profiling in ML and cytokine profiling in plasma. Overall, our results suggest that ML has an important and under-appreciated pathophysiological role in MODS during CI.

L25: Mesenteric Lymphatic Contractile Function in a Rodent Model of Acute Pancreatitis

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Mesenteric lymphatic contractile function can be impaired under inflammatory conditions, such as acute pancreatitis (AP), which can worsen bowel wall oedema and inflammation and contribute to the underlying driver of multi-organ dysfunction. Mesenteric lymphatic contractile dysfunction in inflammatory states is thought to be a direct effect of circulating inflammatory molecules. However, dysfunction at the level of the initial lymphatics within the bowel wall is less well described.

A series of experiments were carried out to determine whether mesenteric lymphatics and bowel wall initial lymphatics are dysfunctional in a rodent model of AP with or without aggressive fluid resuscitation (AFR). Using in vivo video analysis, mesenteric lymphatic contraction frequency, amplitude, and speed decreased in AP, and did not improve with AFR. AP-AFR rats showed signs of developing bowel wall oedema. Despite this, they had significantly lower diastolic filling speeds than sham rats, suggesting the presence of initial lymphatic dysfunction in the bowel wall. This was associated with bowel hypomotility, which likely contributed to the initial lymphatic dysfunction. AP-associated ascites fluid was abundant in pancreatic enzymes and inflammatory cytokines. However, it did not inhibit mesenteric lymphatic contractile function when infused intra-peritoneally into sham rats. An ex vivo mesenteric lymphatic system has been setup to test this further and will be used to test drug candidates for therapeutic enhancement of lymphatic contractile function. Targeting bowel wall motility or mesenteric lymphatic contractile function is a novel therapy for bowel wall oedema.

L26: Advanced Materials to Treat Organ Failure via Inhibition of Pancreatic Enzymes in the Gut

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Introduction: Acute and critical illnesses (ACIs) are typically managed in emergency and intensive care settings in hospitals. More than 20 million patients die per year worldwide from ACIs, predominantly due to progression to organ failure. Currently, there are no specific and effective treatments to mitigate organ failure in ACIs. Recently, the 'gut-lymph model' of organ failure in ACI has demonstrated that 'toxic factors' from the gut enter the lymph and the blood circulation in ACIs, promoting systemic inflammation and organ dysfunction/failure. The major 'toxic factors' in the gut-lymph appear to be pancreatic lipases and proteases.

Aim: we investigated whether delivering small molecule inhibitors (e.g. lipase inhibitor orlistat) and/or adsorbent materials into the gut lumen could bind and reduce pancreatic enzyme activity in the intestinal fluid and/or gut lymph, and potentially mitigate organ failure in ACI.

Method: The in vitro loading and inhibition of pancreatic enzymes by different adsorbent materials, including mesoporous silica particles (MSPs) with different pore sizes (4-12nm), and activated charcoal, were tested. In vivo inhibition of pancreatic enzymes was assessed following infusion of adsorbent materials into isolated intestinal segments in rats. Finally, the efficacy of the adsorbent materials and small molecule enzyme inhibitors was tested in a rodent acute pancreatitis model.

Results: The adsorbent materials (pore size ≥6 nm) successfully adsorbed pancreatic enzymes (>50 mg/g) in a buffer medium. In addition, MSPs (pore size ≥ 10nm) and activated charcoal (≥10 mg/mL) could significantly reduce trypsin and lipase concentrations in the rat intestine lumen. Finally, improvements in serum biomarkers of organ damage were achieved in the groups treated with pancreatic enzyme inhibitors.

Conclusion: Overall, this study found that adsorbent materials can inhibit pancreatic enzymes in the gut and gut lymph, and may provide a novel medical approach to reduce the progression of ACI to organ dysfunction and failure.

L27: Lymphatic Delivery Using Novel Lipid Conjugated Brush PEG Polymers that Hitchhike on Lipid Trafficking Pathways

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Introduction: Lymphatic diseases can be better treated with lymph-directed therapies. Several approaches have been used to increase lymphatic delivery, including encapsulation in nanoparticles, and 'hitchhiking' on endogenous albumin trafficking pathways by conjugating vaccines to albumin binding moieties such as lipids. We evaluated the effect of lipid functionalisation of brush PEG polymer delivery systems on their integration into lipid trafficking pathways, lymph uptake, plasma pharmacokinetics (PK) and biodistribution.

Methods: We synthesised a range of novel brush PEG polymers conjugated with lipids (single short tail (1C2-PEG), single medium tail (1C12-PEG), two medium tails (2C12-PEG), two long tails (2C18-PEG) and cholesterol (Cho-PEG)) and cyanine-5 (to enable quantitation). Association of the polymers with albumin and lipoproteins was assessed using an ultracentrifugation assay. Plasma PK, thoracic lymph and lymph node uptake were compared following IV and SC injection to cannulated rats.

Results: In vitro association with albumin was in the order: 2C12-PEG > 2C18-PEG > Cho-PEG > 1C2-PEG and 1C12-PEG. All polymers associated with various lipoproteins. 2C12-PEG, Cho-PEG and 2C18-PEG polymers had longer plasma half-lives (10-13 hours) than the other polymers (2.6-3.3 hours), consistent with their higher albumin binding. Lymphatic transport was higher for 2C12-PEG polymer (28% of bioavailable dose) than other polymers, consistent with high albumin and lipoprotein binding. Unexpectedly, 1C2-PEG had higher lymph transport than Cho-PEG and 1C12-PEG, which might be explained by its association with low density lipoproteins (LDL).

Conclusion: This study demonstrates, for the first time, that lipidated materials can enter lymph by 'hitchhiking' on lipoproteins as well as albumin. Lipid brush PEG polymers can be specifically engineered to achieve different rates and extents of lymph uptake, plasma PK and biodistribution. These properties will be useful to deliver various therapeutics and vaccines into the lymph.

L28: Adjuvant AS01 Activates Subcapsular Sinus Macrophages and Dendritic Cells in Human Lymph Node Explants Independent of Donor Age

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To progress vaccine development, it is crucial to understand how vaccine adjuvants work in humans, however this is usually studied in animal models. To address this gap, we have developed a novel *in situ* explant model utilizing adult human lymph nodes (LN) to investigate mechanisms of adjuvant action in this key organ where vaccines trigger immunity.

In this study, slices of explanted human skin-draining LNs were exposed to vaccine adjuvants to examine the early innate immune responses that occur when vaccine flows freely to the LN. This human model, utilizing intact LN slices, uncovered responses that were not discernible in LN cell suspensions.

The liposome-formulated adjuvant AS01, part of the highly effective licensed vaccines for herpes zoster and respiratory syncytial virus (Shingrix and Arexvy, GSK), was compared with TLR ligands R848, MPL, and Pam2Cys using this model. Liposomes were preferentially taken up by subcapsular sinus-lining macrophages, as well as monocytes and dendritic cells. In contrast to R848, that directly activated isolated LN dendritic cells to upregulate T cell co-stimulatory molecules and secrete pro-inflammatory cytokines, an intact LN was required for AS01 to activate dendritic cells, suggesting that the LN structure and spatial arrangement of immune cells is critical. The strong pro-inflammatory cytokine response induced by AS01 in intact LN slices was largely unaffected by advanced age, unlike R848. This is an interesting correlate with the age-independent efficacy of Shingrix, due to AS01.

This novel human LN explant model serves as a new tool for studying the mechanism of action of adjuvants in humans and for screening novel formulations to streamline vaccine development.

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L29: The Gravity of the Lymphatic Situation

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Gravity, as a constant force experienced on Earth, exerts a significant influence on the lymphatic system. The lymphatic vessels, which form an extensive network throughout the body, rely on rhythmic contractions of smooth muscle cells to propel lymph fluid against the force of gravity. Changes in gravitational forces, such as during upright posture or microgravity conditions, can alter the normal pumping mechanisms of lymphatic vessels, leading to changes in fluid transport and lymphatic drainage.

The lymphatic system thus responds to changes in gravity-exposure. During upright posture, lymphatic vessels in the lower extremities are subjected to increased hydrostatic pressure, resulting in enhanced contractility to maintain fluid balance. Conversely, in microgravity environments, such as during space travel, the lack of gravitational pull leads to reduced lymphatic pumping, causing fluid stagnation and even impaired immune responses. Understanding these adaptations is the subject of renewed interest as space travel becomes a tourist activity.

This talk explores some of the interactions between gravity and the lymphatic system.