

# **LYMPHATIC IMPAIRMENT FOLLOWING HEART TRANSPLANTATION**

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**LYMPHATIC IMPAIRMENT FOLLOWING HEART  
TRANSPLANTATION**

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*To my dad, Carl R. Ginn Jr.: We did it. I wish you were here to see this come to fruition but know you will always be watching from heaven. Your influence has carried into every moment of this PhD and will continue every day for the rest of my life. I will love and miss you always.*

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## LIST OF SYMBOLS AND ABBREVIATIONS

aAo	Abdominal Aorta
ACR	Acute Cellular Rejection
aIVC	Abdominal Inferior Vena Cava
APC	Antigen Presenting Cells
$\alpha$ -SMA	Alpha Smooth Muscle Actin
BALT	Bronchus-Associated Lymphoid Tissue
CAV	Cardiac Allograft Vasculopathy
CMV	Cytomegalovirus
CNI	Calcineurin Inhibitor
CVD	Cardiovascular Disease
D1	First Diagonal Branch
EMB	Endomyocardial Biopsy
F344	Fischer Rats
HAHT	Heterotopic Abdominal Heart Transplantation
H&E	Hematoxylin and Eosin
HF	Heart Failure
HTx	Heart Transplantation
HLA	Human Leukocyte Antigen
IACUC	Institutional Animal Care and Use Committee
IAL	Inflammation-Associated Lymphangiogenesis
IM	Intramyocardial
ISD	Immunosuppressive Drug

IV Intravascular  
IVC Inferior Vena Cava  
ISHLT International Society of Heart and Lung Transplantation  
LAD Left Anterior Descending Coronary Artery  
LCx Left Circumflex Coronary Artery  
LEC Lymphatic Endothelial Cell  
LEW Lewis Rats  
LM Left Main Coronary Artery  
LMC Lymphatic Muscle Cell  
LV Left Ventricle  
MHC Major Histocompatibility Complex  
MI Myocardial Infarction  
MI/R Myocardial Ischemia Reperfusion Injury  
MMF Mycophenolate Mofetil  
mTOR Mammalian Target of Rapamycin  
NK Natural Killer Cells  
OM1 First Obtuse Marginal Branch  
PA Pulmonary Artery  
pAMR Pathological Antibody Mediated Rejection  
PDPN Podoplanin  
PGD Primary Graft Dysfunction  
PROX-1 Prospero Homeobox Protein 1  
R2 Right Ventricular Branch  
R3 Right Acute Marginal Artery  
RCA Right Coronary Artery

RV Right Ventricle  
SLO Secondary Lymphoid Organ  
SVC Superior Vena Cava  
TLO Tertiary Lymphoid Organ  
tAo Thoracic Ascending Aorta  
tIVC Thoracic Inferior Vena Cava  
VEGF Vascular Endothelial Growth Factor

## SUMMARY

The leading cause of chronic cardiac transplant rejection—coronary artery vasculopathy (CAV)—remains a major factor limiting long-term survival in heart transplant patients worldwide. The disease causes concentric intimal thickening along the coronary arteries leading to luminal narrowing and increased vascular resistance. CAV pathophysiology is thought to involve chronic inflammation and various immunogenic factors, implicating the lymphatic network as a potential therapeutic target.

Lymphatics play a role in every vascularized organ yet are often overlooked in research as a potential therapeutic target. Their ability to modulate tissue homeostasis and immune cell trafficking holds promise in influencing a variety of disease states. Heart transplantation is particularly intriguing as the lymphatic network is severed upon donor heart excision and not surgically reconstructed. The consequence resulting from severed lymphatic vessels in transplanted hearts is unknown.

In this thesis, we aimed to address gaps in cardiac lymphatic research from both a clinical and basic science perspective. Our clinical study investigated lymphatic variations in transplant patients with and without CAV to assess the impact of lymphatics on metrics of transplant rejection and survival. These data correlated lower lymphatic areas to higher mortality in this patient population. Our findings validate lymphatics as a promising biomarker to evaluate mortality risks or even organs prior to procurement. We believe our work will not only stimulate new avenues of research in this field, but also inspire reflection on current surgical techniques, immune suppression, and organ procurement processes.

The outcomes of our clinical study motivated us to do more detailed investigations through animal modeling. We established a heterotopic abdominal heart transplant model (HAHT) to assess longitudinal changes in lymphatic vasculature after HTx and its effect on graft function and survival. These data demonstrated significant increases in lymphatic number and luminal area for extended periods of time post-HAHT and some degree of functional lymphatic drainage at day 14. The period between day 14 and 28 was identified as a critical turning point in pathologic cardiac remodeling with vast therapeutic implications. However, it remains unclear if graft decline during this period was due to increased antigen presentation to secondary lymphoid organs initiating the rejection process or the inability of a bottle-necked lymphatic transport system to rescue the effects of long-term pro-inflammatory cell and interstitial fluid accumulation. These findings improve our understanding on lymphatic adaptations after HAHT but more importantly provide a characterized platform to study induced lymphangiogenic responses intended to rescue transplant function and enhance survival.

In conclusion, we have proven lymphatics to be an important marker and expanded our knowledge of lymphatic biology after HTx. Understanding pathologic conditions associated with lymphatic dysfunction in HTx will provide novel therapeutic targets that enhance the longevity of donor grafts and reduce mortality among the transplant population. Future directions of this work include but are not limited to expanding the clinical study to encompass other prominent transplant diseases and developing a localized, sustained lymphangiogenic-focused therapy to combat the implications of transplant rejection.

# CHAPTER 1. INTRODUCTION

## 1.1 Motivation

Coronary artery vasculopathy (CAV) is one of the most prominent causes of death for long-term heart transplantation (HTx) recipients.<sup>1-3</sup> While medical advances have improved acute heart transplant rejection through immunosuppressive therapeutics, CAV remains the leading cause of chronic transplant rejection and a major factor limiting long term survival.<sup>4-6</sup> CAV is characterized by diffuse intimal thickening of the coronary arteries leading to luminal narrowing and blood flow restriction.<sup>1,6,7</sup> Therapeutic strategies are adept at suppressing acute rejection responses but remain insufficient at inhibiting chronic immune mechanisms.<sup>6</sup> A heavy reliance on long-term immune suppression also compounds the risk of malignancy and infection over time.<sup>8</sup>

There is a growing interest in non-immunosuppressive therapies geared towards targeting cardiac injury, graft survival, and immune tolerance. Some current research is focused on stem cell-based therapies<sup>9</sup>, targeted immunomodulation<sup>10</sup>, anti-fibrotic therapies<sup>11</sup>, and gene therapies<sup>12</sup>. These therapies attempt to target well known underlying mechanisms of ischemia reperfusion (I/R) injury, inflammation, remodeling, and rejection. Unfortunately, very little is known about the causative mechanisms of CAV. CAV is a more complex disease that may need a broader approach to address multiple mechanisms to be successful. Immunologic and non-immunologic risk factors of CAV trigger an inflammatory cascade, vascular cell proliferation, fibrosis, and remodeling.<sup>2,13,14</sup> Inflammatory mediators especially are thought to play a critical role in CAV pathology, implicating the lymphatic network as a potential therapeutic target. The lack of curative

therapies motivates our investigation on whether lymphatic vasculature influences graft function and survival.

Functional lymphatic drainage inherently modulates cardiac function by maintaining the immune response and tissue-fluid homeostasis.<sup>15,16</sup> During HTx, the lymphatic collecting vessels are severed at the time of donor heart excision and not surgically reconstructed in the recipient. The effects of lymphatic disruption and maintenance after surgical insult remain elusive in the context of HTx. The main lymphangiogenic pathway is driven through the vascular endothelial growth factor (VEGF) family, where VEGF-C/VEGFR-3 signaling induces lymphatic endothelial cell migration, proliferation, and differentiation.<sup>17</sup> Previous studies have demonstrated the beneficial effects of augmenting lymphatic growth via VEGF-C/VEGFR-3 signaling by simultaneously enhancing immune clearance and reducing myocardial edema and fibrosis in a disease model of myocardial infarction (MI).<sup>18-22</sup> While these studies build the foundation for pro-lymphangiogenic therapies improving functional cardiac outcomes, the effects of lymphatic augmentation after heart transplantation are currently unknown.

The objective of this dissertation is to characterize lymphatic quantity, structure, and function after HTx to better understand the impact that lymphatic dysfunction has on graft function and long-term survival. We hypothesize that regenerative variability of lymphatics after HTx may affect long-term survival and that lymphatic dysfunction could exacerbate transplant outcomes by preventing the egress proinflammatory cells and stagnation of interstitial fluid in the myocardium. This body of work provides the technical innovation and scientific foundation needed to validate lymphatics as an important

contributor to graft survival, where future studies are poised to introduce targeted lymphangiogenic therapies for CAV treatment and prevention.

## **1.2 Specific Aims**

**Specific Aim 1:** Examine the longitudinal relationship between cardiac lymphatics, vascular remodeling, and survival in human HTx recipients

We hypothesized that variation of cardiac lymphatic remodeling after HTx may be associated with graft function and survival. The study cohort was designed to encompass transplant patients with and without CAV to examine the effects of cardiac lymphatics on late allograft dysfunction. Routine endomyocardial biopsies served to identify differences in lymphatic vasculature over time, where lymphatic quantification allowed data stratification into groups of low and high lymphatic areas. Key characteristics we sought to correlate included survival, donor and recipient demographics (i.e. age, gender, race), graft function (ejection fraction, cardiac output, pressures etc.), rejection episodes, cause of death, and CAV severity.

**Specific Aim 2:** Determine the impact of HTx on lymphatic vasculature in a rodent model

We hypothesized that the disruption of normal cardiac lymphatic flow post-transplantation would exacerbate transplant outcomes by impeding the egress of proinflammatory cells and interstitial fluid leading to reduced cardiac function and survival. We successfully established a heterotopic abdominal heart transplant model (HAHT) in rats to study the long-term changes in lymphatic quantity, structure, and size after surgical insult. In tandem, we developed an assay to assess time-dependent

fluctuations in lymphatic flow that narrowed down the timeframe of systemic lymphatic reconnection. We also tracked pathological remodeling over time through various histologic stainings and quantification techniques.

### **1.3 Significance**

In aim 1, we identified a correlation between lower cardiac lymphatic area and increased mortality after HTx. The lymphatic quantification technique presents a new biomarker capable of assessing long-term survival risks. This novel finding promotes reflections on current surgical techniques, immune suppression, and organ procurement processes geared toward better preservation of extracardiac lymphatic connections and their restorative potential. The conclusion of this clinical study provided experimental justification to investigate a more modifiable animal model of HTx in greater detail. Aim 2 characterized lymphatic vasculature and cardiac remodeling after HAHT over 28 days. That being said, the long-term goal of this project is to elucidate the influence of lymphatic dysfunction after HTx. Lymphangiogenic therapies hold promise in the vast majority of disease states by influencing pathologic edema, inflammation, and remodeling. Understanding how modulation of the lymphatic network influences disease development could diversify current treatment regimens and provide a modality to influence many underlying factors of rejection simultaneously. The data provided within should generate new avenues of research to improve outcomes and quality of life for the transplant community at large.

## CHAPTER 2. LITERATURE REVIEW

### 2.1 Heart Failure (HF)

Cardiovascular disease (CVD) is the leading cause of death worldwide.<sup>23,24</sup> The World Health Organization reported CVD to be responsible for an estimated 32% of all global deaths translating into almost 18 million deaths annually.<sup>23,24</sup> Several factors contribute to CVD prevalence including but not limited to the aging population, medical risk factors (i.e. hypertension, obesity, smoking, physical inactivity, etc.), and the modern sedentary lifestyle.<sup>23</sup> These factors also directly relate to complications of CVD where heart attack, stroke, heart failure (HF), and sudden cardiac arrest are prominent and often fatal.

Specifically, HF is one of the most common and morbid complications that arise from CVD with 1 in 4 people being affected in their lifetime.<sup>25</sup> An estimated 6.7 million Americans over the age of 20 currently have HF and a projected 8.5 million Americans will be affected by 2030.<sup>25</sup> HF occurs when the heart is unable pump blood effectively and meet the demands of the body. This complex disease results from any structural or functional deficiency in ventricular filling or ejection of blood.<sup>26</sup> These impairments can be caused by ischemic heart disease, hypertension, familial/genetic cardiomyopathies, cardiotoxicity, and even substance abuse.<sup>26</sup> The two types of HF include systolic and diastolic failure. Systolic failure or HF with reduced ejection fraction happens when the heart muscle is weakened and can no longer supply the body with enough blood, whereas diastolic failure or HF with preserved ejection fraction occurs when the heart tissue becomes stiff and has difficulty expanding to fill with blood.<sup>27</sup> HF is graded from stage A to D: i) stage A includes patients at risk for HF that do not have symptoms or cardiac

damage but do have significant risk factors, ii) stage B includes ‘pre-HF’ patients that have no symptoms but have evidence of structural or functional heart disease, iii) stage C includes patients with symptoms and signs of HF, and iv) stage D includes patients with marked HF symptoms that consistently interfere with daily life despite optimal clinical management and intervention.<sup>26</sup> Stage D patients present the most advanced form of HF that is commonly dubbed end-stage HF. Currently, the only definitive therapy for end-stage HF is HTx.

## **2.2 Heart transplantation (HTx)**

HTx is a major medical breakthrough intended to restore the quality of life for individuals with end-stage heart failure. The world’s first successful heart transplant was performed in 1967 by Christiaan Barnard in Cape Town, South Africa.<sup>28</sup> Since 1967, there has been significant advancements to HTx medicine. In the United States, the annual rate of HTx has almost doubled from 2,531 in 2013 to 4,545 in 2023.<sup>29</sup> This steady increase in annual rates is projected to continue as the recipient waitlists far exceed the number of annual HTx performed. This rate is further capped by the number of donor organs available and exacerbated by the conservative nature of organ procurement. More than one third of the organs offered are unused partially due to unsuitable donors and the potential risk of early/late allograft dysfunction.<sup>30</sup> Moreover, information on the prevalence of stage D HF patients is scarce; however, it is suspected that this population is far greater than what is clinically reported. With these patients making up the majority of the transplant waitlist, it exemplifies incongruities between recipient demand and donor supply. As a whole, these variables coupled together increase the demand-supply gap making it imperative to develop methods to reduce waitlist and post-transplant mortality.

This life prolonging surgery outweighs the risks associated with HTx and is many people's only option to improve quality of life. With all types of organ transplantation, the new organ is seen by the recipient's immune system as a foreign threat. An organ's specific immune response will vary in both nature and strength. These variations will determine where the organ falls on a spectrum of tolerance. Tolerance-prone organs consist of the kidney and liver, whereas the heart and lungs are more tolerance-resistant.<sup>31</sup> Consequently, clinical treatment regimens for HTx rely on continuous maintenance immunosuppression to prevent rejection but comes with many side effects including increased risk of infection, malignancy, hypertension, high cholesterol, and chronic kidney disease.<sup>3,8,32</sup> Despite these negative side effects, immunosuppressive drugs (ISDs) remain the best treatment strategy to prevent rejection after solid organ transplantation. The transplant community would benefit from novel therapeutic approaches to influence the immune system to improve allograft longevity without long-term suppressive side effects.

The factors affecting HTx survival are both complex and dynamic. It is well documented that current treatment of acute complications surpasses that of chronic complications.<sup>6</sup> National data show survival rates for primary transplants at 1 and 5 years are 90.5% and 77.7%, while repeat transplants were 86.5% and 68.2% respectively.<sup>29</sup> Chronic complications cause mortality to linearly increase with time post-transplantation, where the median time to death or retransplantation is 11.3 years for adults and 14.2 years for pediatric HTx patients.<sup>33</sup> For adult HTx, the incidence of retransplantation was highest among recipients 18-39 years at transplant and lowest among recipients  $\geq 60$  years at transplant suggesting age-related comorbidities reduce the likelihood of retransplantation.<sup>33</sup> Unfortunately, adults are far less likely to undergo retransplantation

compared to pediatric patients due to the increased incidence of complications that make them poor candidates. Understanding factors associated with HTx prognosis and their interactions are imperative to extending survival. Many of these factors are known (i.e. recipient and donor demographics, pre-existing medical risk factors, surgical complications, and infection)<sup>3</sup> and are mitigated in part by careful donor and recipient selection, and advancements to ISDs and infection treatment strategies.

### *2.2.1 Therapeutic Strategies*

Outcomes of solid organ transplantation drastically improved with the introduction of ISDs but only incremental improvements to ISDs have been made in recent decades. The most accepted immunosuppressive regimen involves combinatorial administration of calcineurin inhibitors (CNIs), antimetabolites, antiproliferative agents, and steroids (Table 1).<sup>34</sup> Each type of ISD targets a different segments of the immune system and can affect the cardiovascular system in different ways. While it is well document that ISDs can increase risk of infection and malignancy, these drugs can also cause left ventricular (LV) hypertrophy, myocardial fibrosis, arrhythmia, hypertension, and dyslipidemia.<sup>8</sup>

The main CNI players, cyclosporine and tacrolimus, inhibit T-cell activation by binding to intracellular immunophilins.<sup>35</sup> Cyclophilin A binds cyclosporine and FK-binding protein-12 binds tacrolimus effectively inhibiting calcineurin found on T lymphocytes that prevents T-cell activation. Current regimens favor tacrolimus over cyclosporine since tacrolimus has been shown to reduce likelihood of acute rejection and has potential to reinnervate nerves.<sup>36</sup> Both CNIs described are known to cause

nephrotoxicity and LV hypertrophy warranting a reduction in dose without compromising immunosuppressive efficacy through co-administration with other drugs.<sup>8,36,37</sup>

Mycophenolate mofetil (MMF) is an antimetabolite that was developed to be more potent and selective than azathioprine. MMF inhibits DNA and RNA production and is designed to block the *de novo* synthesis of purine that is required for T- and B-cell proliferation.<sup>35</sup> This process is successful in reducing antibody production and decreasing generation of cytotoxic natural killer (NK) cells and delayed-type hypersensitivity response without having a significant effect on hematopoietic or neutrophil populations.<sup>35</sup> Rodent models of myocardial ischemia reperfusion (MI/R) and myocarditis revealed MMF was capable of preventing apoptosis and LV dysfunction.<sup>38,39</sup>

Mammalian target of rapamycin (mTOR) inhibitors including sirolimus and everolimus are antiproliferative agents that affect downstream pathways of cell growth, proliferation, and survival.<sup>8</sup> Sirolimus, also known as rapamycin, binds to a kinase disrupting cell cycle regulation by preventing the translation of mRNA.<sup>35</sup> Since this cell cycle is arrested, T-cell proliferation is prevented. Everolimus is a synthetic derivative of sirolimus with the same mechanistic target but experiences higher bioavailability and a shorter half-life.<sup>40</sup> Both have been shown to regress or attenuate LV hypertrophy and prevent fibrosis in clinical studies.<sup>41,42</sup>

Steroids are among the first agents used for immunosuppression and continue to have an integral role in rejection regimens. Prednisone is a corticosteroid that binds to glucocorticoid receptors triggering their activation and altering expression of genes involved in inflammatory responses.<sup>43</sup> Various types of leukocytes are affected in number,

distribution, and function as a result. Steroids have a significant amount of side effects including but not limited to hypertension, wound healing, weight gain, diabetes mellitus, and even growth retardation in pediatric patients.<sup>43</sup> To combat this, steroid doses tend to taper over months and have been shown to provide an initial response to 80-85% of rejection episodes.<sup>43,44</sup>

Overall, no single ISD has the capability to prevent rejection in solid organ transplantation. Optimal results are combinatorial and dynamic in the type of drug and dose post-transplantation based on patient status. The negative effects of certain ISDs are intentionally offset by incorporation of other agents and/or a reduction in dosing. It is clear that ISD administration is extremely patient specific and still comes with a fair amount of challenges. Further investigations into novel therapeutic strategies are necessary to reduce the risks associated with chronic immune suppression and improve survival after HTx.

**Table 1. Common immunosuppressive drugs used in combination to prevent transplant rejection.**

<b>ISD Classification</b>	<b>Medication</b>
<b>Calcineurin Inhibitors</b>	Cyclosporine
	Tacrolimus
<b>Antimetabolites</b>	Mycophenolate Mofetil
	Azathioprine
<b>mTOR Inhibitors</b>	Sirolimus
	Everolimus
<b>Steroids</b>	Prednisone

### 2.2.2 *Routine clinical surveillance*

Symptoms of transplant rejection are relatively silent due to cardiac denervation amplifying difficulties in detection that can often lead to graft failure, arrhythmias, ischemia, and sudden cardiac death.<sup>1</sup> Routine surveillance for signs of early cardiac allograft rejection contributes to patient-specific treatment strategies promoting allograft longevity. One of the most valuable surveillance tools in diagnosing HTx rejection is obtaining right ventricular (RV) endomyocardial biopsies (EMBs). In addition, patients often have further diagnostic testing such as coronary angiography, echocardiography, blood chemistry profiles, and complete blood counts. The frequency at which these tests are conducted varies by post-operative time, patient condition, and individual center protocols.

Endomyocardial biopsies are conducted via cardiac catheterization and stained to assess inflammatory infiltrate and myocyte damage, where each sample is graded based on regulations set forth by the International Society for Heart and Lung Transplantation (ISHLT, Table 2). The biopsies are collected during an outpatient procedure where an intravenous line is placed into a vein and the bioprome is guided directly into the heart. Bioprome position is confirmed via x-ray or echocardiography and then sections of the heart are removed from the RV septum. The samples are formalin-fixed and embedded in paraffin prior to staining. Hematoxylin and eosin (H&E) staining is utilized for the initial evaluation of tissue morphology and inflammatory infiltrate. The expected inflammatory infiltrate caused by acute cellular rejection includes lymphocytes, macrophages, and eosinophils.<sup>45</sup> Any neutrophil presence is indicative of alternative processes occurring like healing ischemic injuries, antibody mediated rejection (AMR), or infection.<sup>45</sup> Similarly,

presence of plasma cells suggest a Quilty lesions, healing ischemic injury, or a lymphoproliferative disorder.<sup>45</sup> Myocyte damage is characterized by irregular myocyte borders and distortion of normal myocyte architecture.<sup>45</sup> Myocyte injury is typically accompanied by the infringement of inflammatory cells near myocyte border regions resulting in an irregular scalloped shape and are seen in both early and late ischemic injuries.<sup>45</sup> This standardized histologic assessment tracks immune responsiveness, in turn predicting risk levels of cardiac vasculopathy. This knowledge directly affects treatment regimens but in some cases may not be sufficient alone to diagnose rejection or its severity.

Pathologic antibody-mediated rejection (pAMR) can also be assessed by immunofluorescent staining of C4d on the EMBs. C4d is a complement fragment formed during complement activation that is commonly associated with donor-specific anti-human leukocyte antigen (HLA) antibodies in the serum.<sup>46</sup> Positive C4d staining occurs when antibodies attack the donor heart resulting in the activation of the complement cascade and deposition of C4d around the endothelial layer.<sup>47</sup> However, C4d signal is not an all-encompassing diagnosis of pAMR as even in the absence of C4d signal pAMR may be occurring. C4d staining is usually complemented with CD68/CD31 double staining to further diagnose AMR by identifying the presence of macrophages in the blood vessels.<sup>47</sup> These combined stains more effectively quantify the percent of intravascular macrophages and improve the diagnostic accuracy of pAMR.<sup>47</sup>

There are both invasive and non-invasive modalities to aid in identifying lesions or vascular remodeling in the coronaries over time. Coronary angiography is an invasive diagnostic that is usually used to screen for CAV. CAV is a chronic disease that causes the concentric narrowing of the coronary arteries leading to blood flow restriction. While

coronary angiography is the widely available and a standardized screening technique for CAV, it is limited in its ability to detect early and/or diffuse disease.<sup>48</sup> This technique is proficient in assessing the lumen of epicardial coronaries but has interobserver variability during grading and does not image the vessel wall.<sup>48</sup> Current research involving optical coherence tomography and near-infrared spectroscopy are focused on the development of more sensitive diagnostic tools for earlier and more accurate detection.<sup>48</sup>

### *2.2.3 Acute complications*

With any surgery, comes a host of potential complications. The complications that occur within days post-transplantation are usually surgery-related or primary graft dysfunction (PGD). Surgery-related complications can range widely including bleeding, sternal dehiscence, acute mediastinitis, deep venous thrombosis, pulmonary embolism, and much more.<sup>49</sup> Meanwhile, PGD usually occurs within 24 hours post-HTx and presents as left, right, or biventricular dysfunction with no other identifiable causes.<sup>50</sup> PGD is the leading cause of early mortality post-HTx but the true impact is lessened by contentious diagnosis standards.<sup>50</sup> Individual transplant centers use different criteria and cardiac parameters to diagnose PGD making multi-center comparisons difficult for standardized treatment regimens to be established. Single center studies have published PGD prevalence to range from 2.3% to 28.2% depending on the diagnosis criteria used.<sup>51-55</sup> Generally, PGD is defined as severe ventricular dysfunction leading to cardiogenic shock and circulatory support.<sup>55</sup> PGD etiology is believed to be multifactorial linking many donor- and recipient-related factors to worsened outcomes but further investigations are necessary to elucidate the underlying mechanisms.<sup>55</sup>

Rejection occurs when the recipient's immune system attacks the allograft after identifying it as foreign. T-cell recognition initiates the alloimmune response through direct or indirect pathways. HLAs are expressed by donor cells and recognized by the recipient's T-cells in the direct pathway, while peptides derived from the donor HLAs are presented to T-cells by antigen presenting cells (APCs) in the indirect pathway.<sup>56</sup> The direct pathway primarily participates in acute rejection and the indirect pathway has a later onset affecting chronic rejection.<sup>56</sup> The two types of acute rejection are acute cellular rejection (ACR) and pAMR also known as humoral rejection. ACR typically occurs within the first year after HTx and can affect up to 20% of patients during this timeframe.<sup>57</sup> ACR is a lymphocyte mediated process where foreign antigens initiate T-cell activation that recruits effector cells, such as CD8+ T-cells, macrophages, NK cells, and B-cells into the donor graft.<sup>58</sup> These effector cells in turn orchestrate the rejection process.<sup>58</sup> pAMR can affect up to 15% of patients in the first year and is usually seen several weeks or months after HTx.<sup>45</sup> pAMR involves the indirect recognition of alloantigens by recipient CD4+ T-cells, in turn producing de novo antibodies that target allo-major histocompatibility complex (MHC) expressed on the donor graft.<sup>59</sup> Both ACR and pAMR are considered a continuous process among the alloimmune response.<sup>60</sup> The type of acute rejection seen is dependent on the dominance of different components with respect to time post-transplantation. Treatment regimens consist of increasing ISD doses and adding different ISDs especially steroids. Severe and persistent rejection episodes can call for more drastic measures like plasmapheresis or re-transplantation.

#### 2.2.4 *Chronic complications*

Improvement in survival metrics are predominantly related to increased survival over the first year.<sup>61</sup> Long-term complications like malignancy and cardiac allograft vasculopathy continue to account for 35% of all deaths 10-15 years after HTx.<sup>61</sup> Malignancy alone has an incidence rate of 39% at 10 years post-HTx.<sup>62</sup> The duration and degree of ISD treatment is directly proportional to adverse events and post-transplant malignancies.<sup>62-64</sup> Unfortunately, HTx recipients experience higher rates of malignancy compared to other solid organ transplants due to more intense prophylactic ISD use.<sup>62,63,65</sup> Other factors that increase malignancy risks after HTx are advanced age, male sex, preexisting malignancy, and oncogenic viral infections.<sup>62</sup> Some newer ISDs show improved malignancy-related side effects that protect against rejection while simultaneously inhibiting tumour growth.<sup>66</sup> For example, MMF is associated with significantly lower malignancy compared to azathioprine.<sup>62,66</sup> Continual development of new therapeutics with these side effects in mind are imperative to improve long-term survival post-transplantation.

### **2.3 Cardiac allograft vasculopathy (CAV)**

Transplant rejection is a multifaceted process in which immunologic and non-immunologic factors influence graft and patient survival. While the use of immunosuppressive therapeutics have improved acute transplant rejection, the leading cause of chronic HTx rejection—CAV—remains a major factor limiting long-term survival in heart transplant patients worldwide.<sup>4-6</sup> The prevalence of CAV at 1, 5, and 10 years post-HTx was 8%, 29%, and 47% respectively.<sup>67</sup> This disease remains one of the top three

causes of death 1 year after HTx, where CAV accounts for an estimated 1 in 8 deaths.<sup>68,69</sup> CAV is comprised of a collection of vascular changes that incorporate intimal fibromuscular hyperplasia, atherosclerosis, and vasculitis.<sup>70</sup> By definition CAV induces the concentric hyperplasia of the intimal layer along the coronary arteries that leads to luminal narrowing and blood flow restriction.<sup>1,6,7</sup> CAV can sometimes be erroneously characterized as atherosclerosis.<sup>69</sup> However, its etiology is unique from atherosclerosis in the diffuse and concentric nature of the lesions though the two can exist simultaneously.<sup>69</sup> With some pathologic overlap in vessel disease etiology it was imperative for ISHLT to establish a uniform definition of CAV.

Standardized CAV grading is established on the basis of angiographic findings and graft function: i) CAV<sub>0</sub> (negligible) has no angiographic lesions, ii) CAV<sub>1</sub> (mild) has a left main (LM) stenosis <50% or primary vessel/branch stenosis <70% without graft dysfunction, iii) CAV<sub>2</sub> (moderate) has LM stenosis <50% or single primary vessel/isolated branch stenosis >70% without graft dysfunction, iv) CAV<sub>3</sub> (severe) has left main stenosis  $\geq 50\%$ , stenosis >70% in two or more primary vessels, or isolated branch stenosis >70% in three systems.<sup>71</sup> Graft function is determined via ultrasound or invasive hemodynamic measurements. Dysfunction is considered to be a LV ejection fraction  $\leq 45\%$  and/or evidence of restrictive pathology on an echocardiogram (E/A ratio  $> 2$ , isovolumetric relaxation time  $< 60\text{ms}$ , deceleration time  $< 150\text{ms}$ ) or right heart catheterization (right atrial pressure  $> 12\text{mmHg}$ , pulmonary capillary wedge pressure  $> 25\text{mmHg}$ , cardiac index  $< 2\text{L}/\text{min}/\text{m}^2$ ).<sup>1,71</sup> The standardization of CAV nomenclature provided a basis for consistency in diagnoses but is still influenced by clinician-based variability.

### 2.3.1 *Physical manifestations of CAV*

Graft denervation presents a unique diagnostic challenge in HTx patients. Denervation practically eliminates classic symptoms of disease progression like angina pectoris.<sup>1</sup> When CAV progresses into being symptomatic, the disease is usually at advanced stages with irreversible cardiac damage.<sup>1</sup> The first clinical presentations of CAV are heart failure, arrhythmias, and sudden cardiac death in many cases.<sup>1</sup> Patients also tend to experience atypical symptoms such as exertional dyspnea, gastrointestinal distress, diaphoresis, or syncope.<sup>1,72</sup> Since clinical manifestations are unreliable at diagnosing CAV, it is imperative for routine surveillance testing. Coronary angiography is widely available and the standard method to identify CAV but lacks the sensitivity to detect early and diffuse diseases.<sup>73</sup> Advancements in diagnosis and treatment of CAV are required to reduce mortality and re-transplantation long-term.

### 2.3.2 *Suspected etiology of CAV*

Though CAV is a strong mediator of graft failure, very little is known about its causative mechanism driving the resulting pathologic conditions. Consequently, a wide range of conditions are associated with CAV development to varying degrees. Definitive disease-associated risk factors are divided into those specific to organ transplantation and common risk factors known to play a causal role in native heart coronary artery disease. Transplant-specific risk factors for the development of CAV include the number of HLA mismatches or presence of donor specific antibodies, viremia with cytomegalovirus (CMV), and a history of acute organ rejection. In addition, traditional cardiovascular risk factors such as hyperlipidemia, obesity, diabetes, and hypertension are also associated with

CAV.<sup>13,14</sup> However, their causal mechanism is less clear than in coronary artery disease. CAV patients have an irregular endothelial layer triggering vascular cell proliferation, fibrosis, and remodeling; while expression of HLAs initiate a cascade of cytokine, proinflammatory, and growth factor secretions.<sup>2</sup> This endothelial activation stimulates the chronic inflammatory response that poses as a key mediator in CAV pathogenesis.

Both direct and indirect processes of allo-immunity in CAV are initiated by T-cells driving either B-cell antibody production or cytotoxic cellular responses.<sup>3</sup> The direct process involves recognition of donor-specific MHC molecules via recipient-specific T cells, while the indirect process encompasses donor-specific antigens being presented to recipient-specific T-cells via APCs.<sup>3</sup> Furthermore, CAV increases expression of MHC class I antigens on coronary endothelial cells that are detected by CD8<sup>+</sup> T-cells resulting in cytokine secretion (i.e. INF-gamma, IL-6, IL-2, and TNF- $\alpha$ ).<sup>1</sup> This furthers endothelial cell activation and increases MHC class II antigens that stimulate CD4<sup>+</sup> T-cells.<sup>1</sup> Prolonged endothelial activation results in endothelial dysfunction, inflammatory cell accumulation, smooth muscle hyperplasia, and lipid deposition within the coronaries that cause circumferential intimal thickening.<sup>1,2</sup> The resulting endothelial injury stimulates an expansive cascade of tissue repair mechanisms (i.e. proliferation, fibrosis, and remodeling).<sup>2</sup> Alloimmune responses post-HTx are widely understood to predominantly drive the development of allograft rejection and CAV, however autoimmune responses can play an important role as well. Studies have shown T- and B-cell responses to a cardiac tissue-specific protein, cardiac myosin, similar to autoimmune myocarditis.<sup>3</sup> These similarities in autoimmune responses can affect cardiac function and inevitably cause allograft failure.

### 2.3.3 *Current clinical treatment strategies*

Since CAV outcomes are poor after becoming symptomatic, future research should be focused on early prevention and detection. Current management of CAV consists of mTOR inhibitors and statin therapy.<sup>3</sup> Short-term enhancement of sirolimus and everolimus is known to slow CAV progression through antiproliferative properties. Both are shown to reduce CAV-related events but have the disadvantage of increased frequency of renal insufficiency, bacterial infections, abnormal wound healing, anemia, and thrombocytopenia.<sup>1,74-76</sup> Meanwhile, statins have shown to improve survival rates and reduce the incidence of graft vessel disease within 4 years but lack long-term data.<sup>77,78</sup> Despite these findings, mTOR inhibitors and statins remain inadequate in the treatment and prevention of CAV. The multifaceted nature of CAV limits the effectiveness of ISDs at reducing the alloimmune response.<sup>79</sup> Many non-immunologic factors remain outside of the current therapeutic scope and play considerable roles in the induction and progression of CAV. These risks, coupled with an insufficient treatment regimen for CAV, open the door for unique therapeutic strategies attempting to maintain control of the inflammatory environment and factors directly influencing cardiac function. An alternative approach utilizing the lymphatic vasculature is highly innovative and varies from current research focused on T cell function and antigen identification.<sup>80,81</sup>

## **2.4 Animal models for heart transplantation**

There are two primary methods of transplantation in animal modeling, orthotopic or heterotopic transplantation. Orthotopic transplantation occurs when the donor heart is placed in its anatomically correct position, whereas heterotopic transplantation occurs

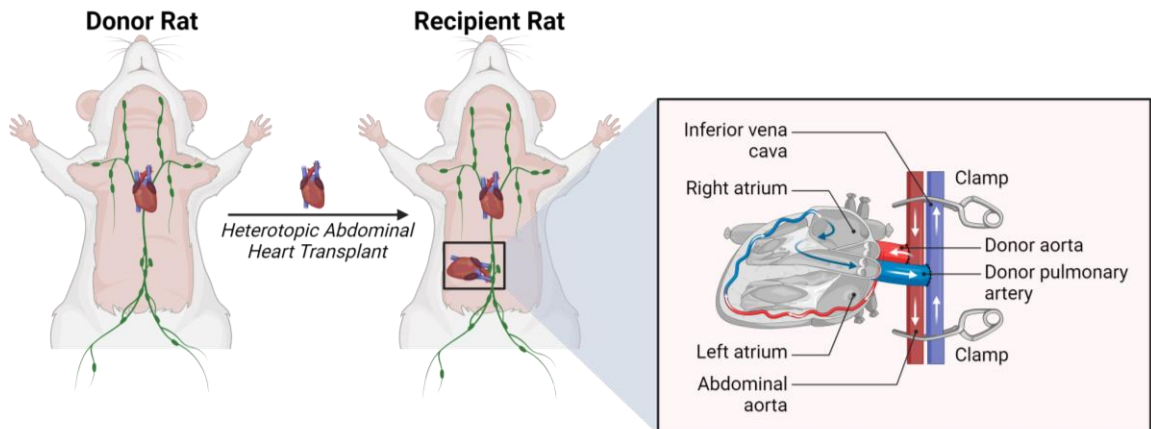
when the donor heart is placed in a location other than the mediastinum.<sup>82</sup> Orthotopic transplantation is the optimal way to study HTx, however it requires the use of cardiopulmonary bypass and larger animals.<sup>82</sup> It is virtually impossible to perform an orthotopic HTx in rodents. On the other hand, heterotopic heart transplantation is widely used in rodents to study ischemia/reperfusion injury, immunosuppression, rejection, and graft coronary vasculopathy.<sup>82-85</sup> The advantage of this model is that the recipient's health is relatively independent of the donor allograft and the vascularized donor organ is more clinically relevant than tissue grafts.<sup>82</sup>

A variety of both large and small animals have been utilized in HTx research. Rats are commonly used due to their size, low model maintenance, reproducibility, and low cost.<sup>82</sup> Taking into account the already enhanced immunogenicity of the perceived foreign donor heart, most transplantology research avoids intra-species variability. Inbred strains are genetically well-characterized and reduce immunologic responses post-transplantation.<sup>82</sup> While inbred strains are currently preferred, recent advances in genetically engineered rats offer a promising modality to improve specificity of data in hypotheses-driven experimentation and outcomes. Large animal models include canines, pigs, and non-human primates.<sup>86</sup> These animals usually have similar anatomical structures and physiologic states to humans and are used to validate outcomes of small animal research to determine clinical applicability. Although non-human primates would be the best animal model to study transplantology research, extreme regulatory standards, high cost, and ethical concerns limit their use. Instead, pigs dominate the field due to their high genomic homology and similarly structured MHC complex with humans.<sup>86</sup>

Our study utilizes a HAHT in rats where the donor organ is implanted in the abdomen distal to the renal arteries and proximal to the aortic bifurcation. HAHT is the classic unloaded heart model where end-to-side anastomoses are made from the donor pulmonary artery (PA) to the recipient abdominal inferior vena cava (IVC) and the donor ascending aorta (tAo) to the recipient abdominal (aAo) (Figure 1). Oxygenated blood flows in through the tAo from the recipient aAo to perfuse the coronary arteries after being diverted by the closed aortic valve. Unoxygenated coronary flow is returned to the right atrium via the coronary sinus and pumped out into the IVC by the RV. The transplanted LV is completely unloaded in this model.<sup>83,87</sup> When unloading was studied in normal rat hearts, they were shown to have contractile function at both the cellular and tissue level after 2 weeks and have decent systolic function out to 4 weeks post-HAHT.<sup>88-90</sup> However, 5 weeks of unloading serves as a turning point of defective fractional shortening and depressed sarcoplasmic calcium uptake capacity.<sup>89,91</sup> These studies demonstrate a time-dependent regression of contractility when subjected to prolonged unloading which can induce atrophy and deterioration of cardiac function long-term.<sup>83</sup> The series of events generated by mechanical unloading in a healthy rat heart provokes similar responses to the failing human heart irrespective of the intended underlying disease.<sup>83</sup> It is reasonable to assume unloading would affect the transplanted heart even more drastically due to its altered hemodynamic parameters.

Two common methods are utilized to monitor the function of the cardiac allografts. Daily palpation of the abdominal wall enables a quick assessment of allograft contractility and weekly electrocardiography offers a more quantitative tool in overall allograft functionality. Tracking the strength of contractions via palpation over time, dubbed

transmitted impulse strength, can give indications of allograft decline.<sup>92</sup> Despite this technically and logistically demanding model, HAHT provides a reproducible platform to study HTx prevention, monitoring, and treatment of acute and chronic rejection.



**Figure 1. Schematic of heterotopic abdominal heart transplantation model and altered hemodynamic flow patterns.**

#### 2.4.1 Animal models of transplant rejection

Both immunologic and nonimmunologic factors affect transplant rejection. The intensity of rejection is dictated by the cumulative strength of the responses to the foreign histocompatibility antigens presented by the donor. Recipient genotype and antigenicity also play a role in response strength to MHC and non-MHC alloantigens. Through these factors, researchers can manipulate study design to induce rejection after HAHT.

Rat models are commonly used with special considerations to strains and gender of the donor and recipient. Chronic rejection has been shown to occur after HAHT between Lewis (LEW) and Fischer (F344) rats, where these inbred strains differ only in minor histocompatibility (non-MHC) loci.<sup>93,94</sup> LEW donors implanted in F344 recipients do not

require the use of immunosuppression and develop diffuse intimal thickening with large and medium sized arteries characteristic of chronic rejection.<sup>94</sup> Processes of allograft vasculopathy involve increased intravascular macrophages, interstitial edema, and neutrophil infiltration.<sup>93</sup> Another study that implanted F344 donors into LEW recipients found early clearance of macrophages via injection of clodronate-liposome was associated with alleviated chronic rejection injuries observed histologically.<sup>95</sup> This suggests that macrophages participate in rejection by potentially acting as APCs or effector cells and releasing cytokines.<sup>95</sup>

Various gender combinations serve to influence rejection in cardiac allografts but tend to require immunosuppression to extend survival.<sup>96</sup> The presence of estrogen in females impacts calcium loading due to hypoxia, nitric oxide availability, vasodilation, fibroblast proliferation, and collagen I and III gene expression.<sup>82</sup> These differences will affect the immunologic and functional outcomes. While donor gender has had no effect, female recipients tend to have significantly shorter graft survival and increased cellular rejection grades.<sup>96</sup> Utilizing these combinations will enable a more comprehensive assessment of transplant biology and eventually therapeutic efficacy in the setting of transplant acceptance and rejection.

Time of impulse decline is the first marker that signifies the clinical manifestation of graft rejection and indicated by a reduction in contractility rating during palpation.<sup>92</sup> The second marker is when the cardiac allograft ceases to contract and denotes the conclusion of the rejection process.<sup>92</sup> During this rejection process, the allograft heart rate and pulsation will decrease linearly while experiencing reduction in size over time.<sup>86</sup> Tracking

the rejection period will provide insights into study design and allow researchers to adjust for longevity of the allograft if needed.

#### *2.4.2 Limitations to existing animal models*

Animal models are monumental in bridging the gap between basic and clinical research, but no animal model is perfect. Many chronic diseases are compressed into an acute timeframe which can distort applications for clinical translation. In turn, aggressive immunosuppressive therapy may be needed to prevent episodes of severe acute rejection and increase the longevity of the allograft.<sup>93</sup> Allotransplantation will always include an immunologic component that requires ISDs to reduce rejection and extend graft survival. Finding the balance between ISDs and the development of new therapeutics can be challenging when the donor organ and recipient are not immunologically compatible. Induction of ISDs to preserve graft function after transplant presents a confounding factor when trying to study transplant immunology in a longitudinal manner.

The surgery itself can significantly compound allograft variability. Regardless of surgeon skill, there will always be a period of ischemia for the transplanted heart. The resulting structural or functional changes will vary by allograft but always remain. Utilizing inbred animals like Lewis rats will minimize the potential immunologic affect but not negate it entirely. Even when there is full MHC compatibility of the donor and recipient, there are differences in the survival depending on the organ transplanted.<sup>82</sup>

HAHT is limited in functionality based on its unloaded nature and non-physiologic blood flow patterns after surgery. While the donor organ basically acts as an aorto-caval fistula with little hemodynamic consequence of the recipient, the alteration in blood flow

restricts aspects of transplantation that can be studied.<sup>82</sup> Moreover, the transplant being unloaded will cause a decline in contractility and accelerate cardiac atrophy over time. These hemodynamic and unloaded parameters account for some features of cardiac function and remodeling to be minimized in clinical translation. Overall, HAHT in rodents still provides the best platform to study rejection and therapeutic interventions for heart transplant recipients regardless of the previously discussed limitations.

## **2.5 Lymphatic System**

The lymphatic system is comprised of highly dynamic structures responsible for regulating flow and homeostasis of every organ in the body. There are multiple tiers of lymphoid organs that contribute to the functionality of the system as a whole. Primary lymphoid organs (i.e. bone marrow and thymus) are responsible for T and B cell production, while secondary lymphoid organs (i.e. spleen and lymph nodes, SLOs) are sites of T-cell maturation, antigen presentation, and initiation of the adaptive immune response.<sup>97</sup> Tertiary lymphoid organs (TLOs) tend to serve as localized immunomodulatory sites in the presence of chronic inflammation.<sup>97</sup>

Lymphatic channels serve as a unidirectional transport system that absorbs interstitial fluid and solutes through lymphatic capillary beds and moves lymph through collecting vessels and lymph nodes to the thoracic duct emptying into the superior vena cava.<sup>16,98,99</sup> This transport system is responsible for trafficking leukocytes, antigen presenting cells, and other soluble antigens that have the potential to impact the vast majority of disease states by influencing pathologic edema, inflammation, remodeling, and

even adaptive immunity.<sup>16,100,101</sup> Lymphatic vessels can be found in every vascularized tissue excluding neural tissue and bone marrow.<sup>16</sup>

Lymphangiogenesis is driven through the VEGF family, where VEGF-C/VEGFR-3 predominates lymphatic development.<sup>17,102</sup> VEGFR-3 is a type of tyrosine kinase receptor that forms homodimers and undergoes autophosphorylation to activate kinase activity when bound to VEGF-C or VEGF-D.<sup>103</sup> VEGF-C/VEGFR-3 signaling is the main pro-lymphangiogenic pathway that induces AKT and ERK activation to regulate lymphatic endothelial cell (LEC) migration, proliferation, and differentiation.<sup>17,99,103</sup> Overexpression of VEGF-C establishes pro-lymphangiogenic effects by providing directional cues for LEC migration and lymphatic vessel augmentation.<sup>104,105</sup> Conversely, anti-lymphangiogenic pathways that suppress lymphatic development include IFN- $\gamma$ <sup>106</sup>, TGF- $\beta$ 1<sup>107</sup>, endostatin<sup>108</sup>, and thrombospondin<sup>109</sup>. IFN- $\gamma$  has been specifically shown to downregulate an essential lymphatic transcription factor, Prospero Homeobox Protein-1 (PROX-1), to influence the homeostatic balance between anti-lymphangiogenic T-cells and pro-lymphangiogenic B-cells.<sup>106</sup>

While the lymphatic system is not officially a part of the immune system, it does play an essential role in immunity. Lymphatics are vital in not only trafficking antigens/immune cells, but are also involved in tissue-fluid homeostasis, antigen presentation, and expression of factors that control local microenvironments.<sup>16,110,111</sup> The ability of the lymphatics to balance plasma-derived fluid and remove excess interstitial fluid determines tissue-specific function and the resulting immune response.

Lymphatic capillary networks with discontinuous button-like junctions, deemed ‘initial lymphatics’, non-selectively uptake interstitial contents (i.e. large macromolecules, cells, fluid, etc.).<sup>102,111,112</sup> Lymphatic collecting vessels clear the lymph downstream via intrinsic lymphatic muscle cell and skeletal muscle contraction into lymph nodes, which provide a central hub for migratory lymphocytes and APCs trafficked from the blood and interstitial fluid.<sup>110-112</sup> Subsequently, incurring activated immune cells and a resulting cascade in the lymph’s return to blood circulation via lymphaticovenous junctions (e.g. thoracic duct or subclavian veins).<sup>102,111</sup> That being said, the lymphatic network directly influences inflammation and plays a pivotal role in its resolution. With consideration to lymphatic function, it is reasonable to assume inflammation would increase lymphatic drainage as a compensatory mechanism attempting to achieve resolution. This inflammation-associated lymphangiogenesis (IAL) is important in removing excess immune cells, harmful antigens, cellular debris, etc.<sup>16,100</sup> Studies have shown that in response to inflammatory stimuli, lymphatic vessels not only expand and grow but also have the ability to incorporate circulating progenitor cells into the growing vessels by transdifferentiating them into LECs.<sup>100,113-117</sup> The complex interplay between lymphatic biology and immunological interactions in the context of many pathological entities remains obscure, but provides a promising target to further investigate.

### *2.5.1 Lymphatics in cardiovascular disease*

The heart is comprised of a rich lymphatic network, but very little is known about the physiologic and pathophysiologic role of cardiac lymphatics. Cardiac lymphatics are developed directly after blood vasculature during embryogenesis and are heavily reliant on the ingrowth of cardinal vein endothelial cell-derived PROX1+VEGFR3+ lymphatic

precursor cells for continued growth.<sup>18,99,118-120</sup> Developmental lymphangiogenesis extends the lymphatic network from the heart's base to apex, predominately traveling along the epicardial surface following the path of the coronary arteries.<sup>99</sup> The route of cardiac lymphatic drainage is thought to flow from subendocardial to subepicardial lymphatics towards the mediastinal lymph nodes under the aortic arch and near the trachea.<sup>18,22,118,121-123</sup> Moreover, the lymphatic vessels near the epicardial surface of the heart are mostly comprised of valved pre-collectors with minimal lymphatic muscle cells (LMCs) to drive lymph propulsion; therefore, lymphatic transport in the heart is dependent on extrinsic factors like cardiac muscle contraction and twisting forces.

The consequence of having impaired cardiac lymphatic flow may explain many unknown pathologic entities with its functionality being critical to many organ systems throughout the body. This gap in knowledge offers a distinctive opportunity to pinpoint the lymphatic system's role in many pathophysiologic conditions, particularly heart transplantation since lymphatic channels are severely disrupted. During heart transplantation, the lymphatic collecting vessels are severed at the time of heart excision and not surgically reconstructed. Immune cells that are continually delivered to the heart via myocardial capillaries, have no functional lymphatic drainage to egress the buildup. This disruption of lymphatic drainage may potentiate inflammation by impeding the egress of immune cells and pro-inflammatory cytokines out of the donor graft. Furthermore, the functionality of this organ is partially reliant on the maintenance of microvascular permeability and the accumulation of myocardial interstitial edema fluid.<sup>124</sup> The lymphatic vasculature plays a substantial role in maintaining organ-specific tissue-fluid homeostasis, in turn compounding the importance of intact and healthy lymphatic vasculature within

transplanted hearts. Therefore, severed lymphatic vasculature may result in a pathological response that can detrimentally affect cardiac allograft function and survival long-term.

### 2.5.2 *Lymphatic-based therapeutics in other cardiac models*

To date, there are no clinically approved therapies targeting cardiac lymphatic transport. Nonetheless, literature has shown reduced or dysfunctional lymphatic vasculature in various cardiac models perpetuates inflammation, myocardial edema, and fibrosis development leading to poorer cardiac outcomes.<sup>125,126</sup> In tandem, unresolved inflammation and edema drive fibrosis development inducing myocardial remodeling that contributes to diastolic and systolic dysfunction.<sup>126</sup> Pro-lymphangiogenic therapies have shown great potential in limiting edema and inflammatory accumulation in various pathological diseases, such as: lymphedema, irritable bowel syndrome, nephropathy, atherosclerosis, MI, and much more.<sup>19,126-130</sup> Specifically, most cardiac lymphatic research is centered around MI injury caused by coronary occlusion that results in reduced blood flow and lymphatic function in the surrounding heart muscle.<sup>19,125</sup> When cardiac damage induces edema buildup, the extracellular matrix proteins connected to lymphatic capillaries pull LECs open to enhance lymphatic drainage capabilities.<sup>126</sup> Moreover, a known cardiac compensatory mechanism consist of increases in lymphatic transport in response to cardiac pathologies that involve myocardial edema.<sup>126,131</sup> This mechanism is supported in more recent studies involving pro-lymphangiogenic therapies proven to enhance immune cell clearance, while reducing edema and fibrosis after MI.<sup>18-22</sup> Conversely, anti-lymphangiogenic therapies (i.e. MAZ-51 inhibition) have been shown to exacerbate disease states by blocking VEGF-C and VEGF-D induced phosphorylation of VEGFR-3.<sup>132,133</sup> These studies indicate administration of pro-lymphangiogenic growth factors

provide a potential avenue to target lymphatic vessels in diseases of every organ in the body.

While the pathophysiologic effects of lymphangiogenesis and the role of VEGF-C/VEGFR-3 signaling has been extensively evaluated in cancer metastasis research,<sup>134-138</sup> it is severely understudied in many other pathologic conditions. With existing pathway knowledge, we are poised to examine the influence of impaired lymphatic drainage on the immune response following cardiac transplantation. A localized source stimulating lymphatic development or reconnection has the promising ability to combat major determinants of allograft survival. Thus, understanding the complex interplay between the lymphatic and immune systems will lead to significant advancements in clinical medicine by providing alternative therapeutic strategies to compensate for ineffective CAV treatment strategies.

### *2.5.3 Lymphatic vasculature in other solid organ transplants*

All solid organ transplants are similar in the lack of lymphatic reconnection during surgery. This lymphatic disruption has the potential to impact allografts in an organ specific manner. For many organs it remains unclear whether lymphatics will be beneficial by promoting immune cell clearance and resolving edema or detrimental by advancing antigen presentation to secondary lymphoid organs (SLOs) initiating the rejection process. The following studies are focused on organ-specific lymphangiogenic effects on allograft function and survival.

Lung allografts differ from cardiac allografts in that rejection is independent of SLOs. Lung allografts provide a sufficient environment for alloimmune activation without

lymphatic involvement of draining lymph nodes to trigger rejection.<sup>139</sup> Lung allografts from a rodent model have demonstrated that T-cells can be primed by dendritic cell interactions outside of SLOs.<sup>139</sup> Despite this fact, disruption of lymphatic vessels in rodent lung allografts led to impaired allograft function and lymphangiogenesis was associated with alleviating rejection.<sup>140</sup> Lung allografts have shown to develop bronchus-associated lymphoid tissue (BALT), a TLO associated with a higher tolerance in states of chronic rejection.<sup>141</sup> BALT-resident FOXP3+ T-cells were found to suppress B-cell production and inhibit AMR, in turn challenging the notion that humoral responses are regulated peripherally.<sup>141</sup> Further investigations into TLO involvement of allograft tolerance would also affect organs like the kidney.

Renal lymphatics not only regulate inflammation but also control pressure, volume, and protein content of the interstitial fluid.<sup>97</sup> Clinical studies have shown a presence of de novo lymphangiogenesis in 61-74% of renal biopsies from kidney allografts.<sup>142</sup> However, there was no significant difference in lymphatic vessel density between healthy renal transplant patients and patients with acute or chronic allograft nephropathy.<sup>142</sup> The difference lie in the location of lymphatic neoangiogenesis and whether it was an area with or without cellular infiltrates. Renal allografts followed out to 1 year were found to function better if lymphatic vessels were located in areas of high cellular infiltrates compared to lymphatic vessel-free infiltrates, which suggests lymphangiogenesis may impact the pathogenicity of the cellular infiltrates found in renal transplants.<sup>142</sup> Moreover, rodent models of renal transplantation have demonstrated allografts overexpressing VEGF-C attenuated rejection and extended allograft survival.<sup>143</sup> Taken together these advances have shown the lymphatic system is capable of either enhancing or repressing inflammation and

therefore disease progression. With inflammation being a key regulator in both acute and chronic rejection for all solid organ transplants, lymphangiogenic therapies are poised to potentially influence survival outcomes. However, the functional role of these therapies may differ based on organ-specific needs and lymphatic heterogeneity.

The study of lymphatics in solid organ transplantation is a novel area of research that has significant clinical implications to improve transplant outcomes. Furthering our understanding of lymphatic dysfunction after transplantation will provide crucial insight into the dynamics that contribute to graft survival, rejection, and long-term function. This research could lead to targeted immunomodulatory therapies to prevent rejection, reduce the need for immunosuppression, and improve the overall success of organ transplants.

## **CHAPTER 3. CLINICAL STUDY: CARDIAC LYMPHATICS PREDICT SURVIVAL AFTER HEART TRANSPLANTATION**

### **3.1 Abstract**

*Introduction:* Functional lymphatic drainage inherently modulates cardiac function by maintaining the immune response and tissue-fluid homeostasis. During HTx, the lymphatic collecting vessels are severed at the time of heart excision and not surgically reconstructed in the recipient. The consequence resulting from impaired lymphatic drainage in transplanted hearts is unknown. This study hypothesized that variations in cardiac lymphatics could influence graft function and survival. *Methods:* RV EMBs from 1 week to 5 years after HTx were evaluated for blood and lymphatic vasculature utilizing various histologic markers. Lymphatic area was quantified throughout the lifespan of cardiac allografts in patients with and without CAV. *Results:* Histology demonstrated the ability to characterize lymphatic vessels from HTx biopsy specimens. When patient cohorts were separated into low and high lymphatic areas at 1 year, patients in the lower lymphatic group were significantly associated with higher mortality. *Translational Impact:* This study brings light to an underappreciated clinical parameter that has the potential to generate new avenues of research to improve outcomes and long-term survival after HTx.

### **3.2 Introduction**

HTx is the definitive therapy for end-stage heart failure. In 2023, 4,545 HTx surgeries were conducted in the United States and an estimated 30,000 Americans are living with a heart transplant.<sup>29</sup> The demand for donor hearts is far greater than the number

of organs available and long-term survival has only minimally improved in recent decades.<sup>13</sup> While acute rejection is clinically apparent and treated with immunosuppression, chronic rejection and allograft dysfunction requires diligent surveillance as current treatment strategies are insufficient. CAV is the leading cause of chronic rejection and one of the top three causes of death for HTx patients after the first year.<sup>68</sup> Unlike acute rejection, CAV increases in prevalence over time eventually resulting in graft failure.<sup>67,68</sup> This disease causes the concentric hyperplasia of the intimal layer that leads to distal tapering of the coronary arteries and blood flow obstruction.<sup>1,6,7,144</sup> Though CAV is a strong mediator of graft failure, very little is known about its causative mechanism as it continues to plague the transplant community. Denervation of the graft further complicates diagnosis by graft dysfunction presenting as asymptomatic without angina until ischemic injury at advanced stages. Its pathology is a complex interplay of both immunologic and non-immunologic factors that usually manifests with a severe and sudden onset (Figure 2).<sup>145</sup> The existence of CAV poses considerable challenges to the long-term treatment of HTx patients, necessitating new paradigms in understanding allograft dysfunction to mitigate progression and improve the survival.

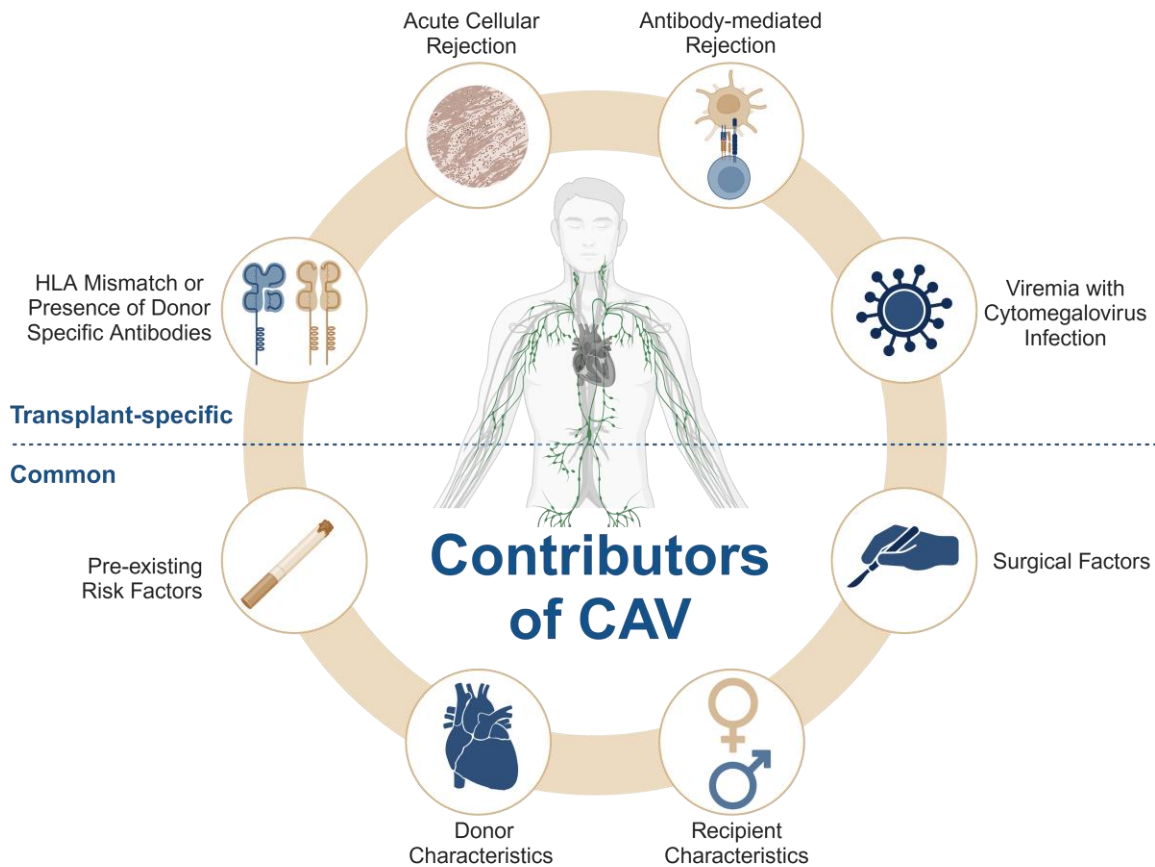
Lymphatic vasculature is comprised of highly dynamic structures that serve as a transport system to absorb interstitial fluid and traffic lymph which affects pathologic edema, inflammation, remodeling, and even adaptive immunity.<sup>16,100,101</sup> The heart contains an extensive lymphatic network essential for cardiac function and healing after injury by clearing immune cells, extracellular fluid, and debris.<sup>15,125</sup> Developmental lymphangiogenesis extends the lymphatic network from the heart's base to apex, predominately traveling along the epicardial surface following a unidirectional path along

the coronary arteries.<sup>99</sup> The route of cardiac lymphatic drainage is thought to flow from subendocardial to subepicardial lymphatics towards the thoracic duct that empties into the superior vena cava.<sup>18,22,99,118,121-123</sup> Proper lymphatic function is imperative for the success of any organ system but not prioritized in surgical techniques and medical management of solid organ transplantation. The lymphatics vessels are severed during excision of the donor graft and never surgically reconnected in the recipient, leaving donor myocardial lymphatics intact but no connections to the recipient lymphatic system after HTx. Disrupting these routes of immune cell trafficking may detrimentally alter local and systemic immunobiology in turn affecting both acute and chronic rejection. Various solid organ transplant studies have shown lymphatic regeneration as early as 3 days after surgery but an extended delay in restoring functional lymphatic drainage depending on the organ.<sup>97</sup> Animal models of renal and lung allografts did not start to experience restoration in flow until after 14 and 12 days post-transplantation respectively.<sup>146,147</sup> Even with the lymphatic drainage seen at these early timepoints, the timeline of the network's full functional capacity remains ambiguous and most likely varies by subject. While the implications of a severed lymphatic system within HTx are unknown, lymphatics ultimately have the potential to determine graft survival by regulating the immune response and tissue-fluid homeostasis.

In the context of HTx, there are controversial studies on whether lymphatics will incite or resolve factors of chronic rejection. The beneficial effects of lymphatic augmentation after injury are well documented in other cardiac animal models, where lymphatic growth post-MI has shown to enhance immune clearance, resolve myocardial edema, and improve cardiac function.<sup>18-22</sup> Conversely, some groups show that blocking

lymphatic activation inhibits the initial immune response and limits antigen presentation to reduce rejection risk factors.<sup>148-150</sup> While these processes are clearly complex, further investigation into lymphatic roles after HTx have the potential to impact both clinical and translational research with new methods of early detection and therapeutic intervention for transplant rejection worldwide.

The lymphatic system is vastly understudied, however determining variations in lymphatic restoration over time have the potential to impact adverse outcomes and patient survival in all solid organ transplants. We hypothesize that lymphatics play a beneficial role in HTx and patients with lower lymphatic amounts are predisposed to higher mortality. This study aims to develop a new method of identifying at risk transplant patients to lay the groundwork in targeting CAV prevention. Identification of enhanced evaluation techniques and new therapeutic avenues targeted to extend graft longevity are essential to improve long-term patient survival, while reducing the need for re-transplantation especially in the pediatric community.



**Figure 2. Schematic of factors known to influence CAV development and progression.**

### 3.3 Methods

The study is approved by Emory University’s Institutional Review Board and informed consent was waived.

#### 3.3.1 Study population and design

This study is a single-center, blinded, retrospective case-control study utilizing EMBS of 50 primary HTx patients from Emory University’s (Atlanta, GA) Transplant Center between June 2010 and May 2017. Our cohort was designed to encompass

transplant patients with late allograft dysfunction and transplant-matched controls. Patients with late allograft dysfunction had the following degrees of CAV severity: i) mild CAV<sub>1</sub> and ii) severe CAV<sub>2-3</sub>. Transplant-matched controls had no noted history of CAV within their medical records. To determine the role lymphatics play in late allograft dysfunction, the cohort included adults that survived at least 5 years after HTx and were greater than 21 years of age. Patients were excluded if they were missing an EMB and/or angiogram at the 1 or 5 year timepoints. Information on recipient demographics, treatment regimen, echocardiograms, angiograms, laboratory results, and survival was obtained from medical records. Non-transplanted control patients that underwent a non-cardiac related fatality were identified to delineate cardiac lymphatic distribution in normal hearts. These non-transplanted controls had no noted cardiac abnormalities on their pathology reports.

### *3.3.2 Histology of endomyocardial biopsies (EMBs)*

EMBs taken in the course of normal clinical care were stained with H&E to identify episodes of ACR. Slides were read by pathologist and reported in the anatomic pathology reports within patient medical records. Pathologist grading is in accordance with the International Society for Heart and Lung Transplantation 2004 guidelines (ISHLT; Table 2).

**Table 2. ISHLT grading guidelines adapted from Stewart et al.<sup>45</sup>**

<b>2004 ISHLT Grade</b>	<b>Description</b>
<b>0R</b>	No rejection
<b>1R, mild</b>	Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage
<b>2R, moderate</b>	Two or more foci of infiltrate with associated myocyte damage
<b>3R, severe</b>	Diffuse infiltrate with multifocal myocyte damage ± edema ± hemorrhage ± vasculitis

3.3.2.1 Immunofluorescence Staining

Slides were deparaffinized by being submerged in xylene for 10 minutes (2x), 100% ethanol for 10 minutes (2x), 95% ethanol for 10 minutes, 70% ethanol for 7 minutes, and 50% ethanol for 7 minutes. Slides were washed in 1x PBS for 5 minutes before being placed in the pressure cooker in citrate acid buffer (pH=6) for 10 minutes. The pressure cooker was allowed to depressurize for 30 minutes prior to transferring the slides to 1x PBS. Permeabilization of the tissue occurred in 0.1% Triton-X100 buffer for 10 minutes. Wash steps were repeated in 1x PBS for 5 minutes (2x). A hydrophobic barrier pen outlined the target tissue to minimize reagent waste. Slides were blocked in 10% goat serum diluted with 1x PBS at room temperature for 1 hour. Primary antibody incubation was performed overnight at 4°C in 2% goat serum diluted with 1x PBS. Primary antibodies included PDPN (1:50, Abcam, ab77854) and  $\alpha$ -SMA (1:200, Abcam, ab5694). Wash steps were repeated with 1x PBS for 5 mins (5x). Secondary antibody incubation included goat anti-mouse

Alexa Fluor 568 (1:1000, ThermoFisher, 11004) and goat anti-rabbit Alexa Fluor 488 (1:2000, ThermoFisher, 11008) in 2% goat serum diluted with 1x PBS at room temperature for 1 hour. Wash steps were repeated with 1x PBS for 5 mins (5x) then tissue was stained with 300  $\mu$ M DAPI (1:1000, Invitrogen, D21490) for 10 minutes. Slides were washed in 1x PBS for 5 minutes and mounted in diamond anti-fade medium (Invitrogen, P36965). Fluorescent images were taken on either the widefield Keyence BZ-X800 or the confocal Zeiss LSM800 microscopes. Quantification of lymphatic vessels was accomplished via ImageJ (National Institutes of Health).

### 3.3.2.2 Masson's trichome staining

Slides were deparaffinized as stated previously and washed in diH<sub>2</sub>O. Slides were then fixed in Bouin's Solution (Sigma, HT10132) at room temperature overnight. Excess Bouin's was rinsed in running tap water then dipped in diH<sub>2</sub>O prior to being stained with Weigert's Iron Hematoxylin solution (Sigma, HT1079) for 5 minutes. Slides were washed, stained in Biebrich Scarlet-Acid Fuschin (Sigma, HT151) for 5 minutes, and washed again. Slides were set in Phosphotungstic/Phosphomolybdic Acid Solution (Sigma, HT152 and HT153), washed, then stained with Aniline Blue Solution (Sigma, B8563) for 4 minutes. Wash steps were repeated followed by placing slides in 1% Acetic Acid for 2 minutes and dehydrating through graded ethanol and xylene. Slides were mounted using cytooseal (Richard-Allan Scientific, 8310-4) and imaged on the NanoZoomer (Hamamatsu Photonics). Percent fibrosis was quantified through color deconvolution and thresholding within ImageJ (National Institutes of Health).

### 3.3.3 *Angiography*

Coronary angiography is routinely performed on transplant patients to assess the development of CAV. Angiograms were reviewed for all patients at or close to the 1 and 5 year EMB collection dates. Since CAV is characterized by the diffuse thickening of the coronary arteries, coronary luminal diameters were measured at proximal, middle, and distal segments (Table 3). Reviewed on a side-by-side basis, exact locations of the LM, LAD, LCx, and RCA were determined for 1 and 5 year timepoints. All measurements were normalized to catheter size and taken at consistent anatomical landmarks from the same angiographic views. Proximal segments were measured at the ostium of every coronary vessel. Middle segments were measured distal to the first diagonal branch (D1) for the LAD, obtuse marginal branch (OM1) for the LCx, and between the right ventricular branch (R2) and obtuse marginal artery (R3) for the RCA. Distal segments were measured as follows: i) halfway between D1 and the end of the LAD, ii) halfway between OM1 and the end of the LCx, and iii) halfway between R3 and the end of the RCA.

**Table 3. Angiographic landmarks were utilized for segmental analysis to enhance measurement specificity in the same angiographic view for 1 and 5 years.**

<b>Coronary</b>	<b>Segment</b>	<b>Measurement Location</b>
<b>LM</b>	Proximal	Ostium of the LM
<b>LAD</b>		
	Proximal	Ostium of the LAD
	Middle	Distal to D1
	Distal	Halfway between D1 and the end of the vessel
<b>LCx</b>		
	Proximal	Ostium of the LCx
	Middle	Distal to OM1
	Distal	Halfway between OM1 and the end of the vessel
<b>RCA</b>		
	Proximal	Ostium of the RCA
	Middle	Between R2 and R3
	Distal	Halfway between R3 and the end of the vessel

#### 3.3.4 Statistical analyses

Biopsy samples were read blinded to CAV status. Continuous variables were expressed as mean  $\pm$  standard deviation (SD) and analysed with either an unpaired t-test or one-way ANOVA. Categorical variables were expressed as a number and percentage that was analysed with  $\chi^2$  testing. Related measure ANOVAs or Kruskal-Wallis tests were performed for continuous variables depending on data distribution and variance. All distributions into low and high lymphatic cohorts utilized the lymphatic median quantified at one year. Kaplan-Meier plot was implemented in conjunction with log-rank test statistics to observe survival percentages between patients with low and high lymphatic quantities. Adjustments were made with cox proportional hazards modeling. All statistical testing was two-sided where p-values of 0.05 or less were considered significant.

## 3.4 Results

### 3.4.1 Study population

Between June 2010 and May 2017, coronary angiograms and EMBs of 50 HTx patients were obtained at consistent timepoints spanning 5 years. Transplant patients were stratified into cohorts based on all-time angiographic diagnosis of CAV per ISHLT guidelines (Table 4): i) no CAV<sub>0</sub> (n=26, 77% male, mean age 49y ± 12), ii) mild CAV<sub>1</sub> (n=12, 83% male, mean age 53y ± 8), and iii) severe CAV<sub>2-3</sub> (n=12, 83% male, mean age 45y ± 10). Patient groups had a higher prevalence of males as is characteristic of the transplant and heart disease population at large (80%). The cohorts as a whole consisted of many patients having multiple baseline medical risk factors like hypertension (n=35, 70%) and diabetes (n=23, 46%). Generally, patients with either mild CAV<sub>1</sub> or severe CAV<sub>2-3</sub> had comparable or higher percentages of diabetes and hypertension; however, the mild CAV<sub>1</sub> cohort had the highest incidence of chronic kidney disease (42%, p = 0.0020) and cancer (25%, p = 0.0134). While not significant, cytomegalovirus (CMV) mismatch did raise in occurrence with CAV severity (15%, 17%, 25%; p = 0.2385) and could be a notable factor in CAV development as stated in other studies.<sup>151</sup> Moreover, severe CAV<sub>2-3</sub> had the highest incidence of total number of rejection episodes graded at both 1R (14.0 ± 5.7; p = 0.0834) and 2R (1.8 ± 2.0; p = 0.1997).

**Table 4. Recipient characteristics based on disease severity.**

Variable	Overall (n=50)	Severe CAV <sub>2-3</sub> (n=12)	Mild CAV <sub>1</sub> (n=12)	No CAV <sub>0</sub> (n=26)	p-value
Transplant Age, y	49 ± 11	45 ± 10	53 ± 8	49 ± 12	0.2605
<b>Gender, n (%)</b>					
Male	40 (80%)	10 (83%)	10 (83%)	20 (77%)	0.8448
Female	10 (20%)	2 (17%)	2 (17%)	6 (23%)	0.4827
<b>Race, n (%)</b>					
Caucasian	23 (46%)	5 (42%)	5 (42%)	13 (50%)	0.5940
Black	27 (54%)	7 (58%)	7 (58%)	13 (50%)	0.6592
<b>HF Etiology, n (%)</b>					
Ischemic Cardiomyopathy	12 (24%)	4 (33%)	3 (25%)	5 (19%)	0.1431
Non-ischemic Cardiomyopathy	38 (76%)	8 (67%)	9 (75%)	21 (81%)	0.5076
<b>Medical Risk Factors, n (%)</b>					
Diabetes	23 (46%)	6 (50%)	6 (50%)	11 (42%)	0.6598
Hypertension	35 (70%)	8 (67%)	10 (83%)	17 (65%)	0.2475
Chronic Kidney Disease	13 (26%)	2 (17%)	5 (42%)	6 (23%)	<b>0.0020</b>
Cancer	8 (16%)	1 (8%)	3 (25%)	4 (15%)	<b>0.0134</b>
CMV Mismatch, n (%)	9 (18%)	3 (25%)	2 (17%)	4 (15%)	0.2385
Ischemic Time, min	218 ± 46	223 ± 59	225 ± 47	214 ± 41	0.5581
<b>Rejection Episodes, #</b>					
1R	11.1 ± 5.9	14.0 ± 5.7	11.6 ± 5.8	9.5 ± 5.4	0.0834
2R	1.2 ± 1.6	1.8 ± 2.0	1.4 ± 1.5	0.8 ± 1.2	0.1997

Donor information was collected from the UNet system and provided by the United Network for Organ Sharing (UNOS; Table 5). Interestingly, the severe CAV<sub>2-3</sub> cohort had the lowest average recipient and donor age (45y ± 10, 23y ± 6) compared to mild CAV<sub>1</sub> (53y ± 8, 28y ± 10) and no CAV<sub>0</sub> (49y ± 12, 28y ± 7) cohorts. Only 2 (4%) females were a part of the total donor pool and both resided in the severe CAV<sub>2-3</sub> cohort (p = 5.7777E-08). Gender mismatching was relatively consistent across cohorts and only comprised 20% of the total population. Lastly, head trauma dominated donor cause of death (n=39, 78%)

and was followed by a relatively even distribution of anoxia from drug intoxication (n=2, 4%), anoxia—other (n=5, 10%), and cerebrovascular events (n=4, 8%).

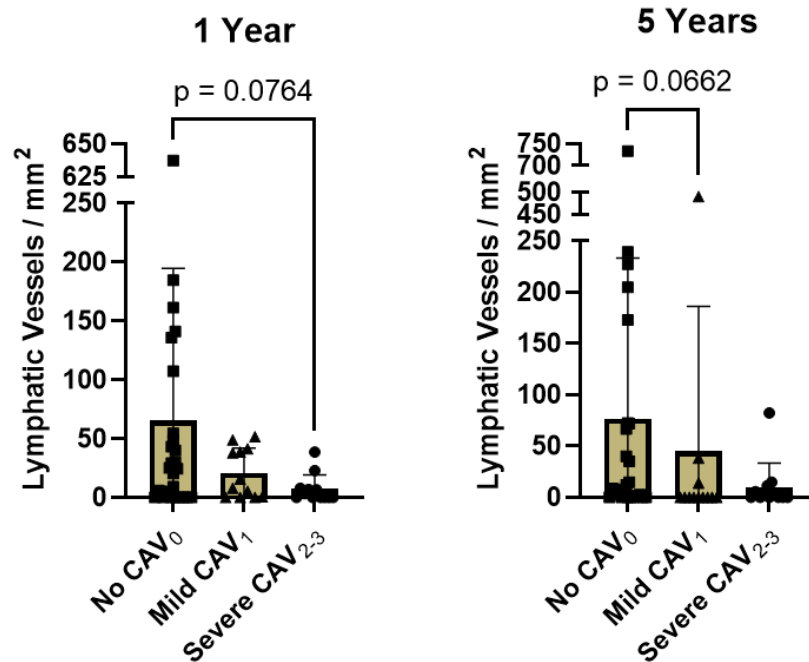
**Table 5. Donor characteristics based on disease severity.**

<b>Variable</b>	<b>Overall (n=50)</b>	<b>Severe CAV<sub>2-3</sub> (n=12)</b>	<b>Mild CAV<sub>1</sub> (n=12)</b>	<b>No CAV<sub>0</sub> (n=26)</b>	<b>p-value</b>
Donor Age, y	27 ± 8	23 ± 6	28 ± 10	28 ± 7	0.2753
<b>Donor Gender, n (%)</b>					
Male	48 (96%)	10 (83%)	12 (100%)	26 (100%)	0.3752
Female	2 (4%)	2 (17%)	0 (0%)	0 (0%)	<b>5.7777E-08</b>
Gender-matched	40 (80%)	10 (83%)	10 (83%)	20 (77%)	0.8448
Gender-mismatched	10 (20%)	2 (17%)	2 (17%)	6 (23%)	0.4827
<b>Cause of Death, n (%)</b>					
Anoxia (Drug Intoxication)	2 (4%)	1 (8%)	0 (0%)	1 (4%)	<b>0.0138</b>
Anoxia (Other)	5 (10%)	2 (17%)	0 (0%)	3 (12%)	<b>0.0004</b>
Head Trauma	39 (78%)	8 (67%)	10 (83%)	21 (81%)	0.3510
Cerebrovascular Event	4 (8%)	1 (8%)	2 (17%)	1 (4%)	<b>0.0123</b>

### 3.4.2 Lymphatic vasculature and survival

Next, we sought to investigate whether variations in lymphatics density were associated with CAV and the accompanied risk factors. Lymphatic vessels were readily identifiable and quantified to obtain the number of lymphatics per mm<sup>2</sup> of tissue in whole heart cross-sections at 1 and 5 years. When stratified by CAV severity (Figure 3), Kruskal-Wallis testing identified trending differences between CAV cohorts at 1 year (p=0.0810) and significant differences at 5 years (p=0.0474). However, Dunn’s multiple comparison testing only revealed nonsignificant trends between specific cohorts at each timepoint. Low

cohort numbers stand to substantially affect significance of the multiple comparison test in this instance.



**Figure 3. Lymphatic area experienced step-wise decrements with increased CAV severity.** Lymphatic vessels per mm<sup>2</sup> were quantified for both 1 and 5 year timepoints and stratified by CAV severity. The statistical analysis performed was a nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test. Error bars indicate SD.

Patients were then stratified into groups of low (n=25, 20% female, mean age 48.4y ± 11.0) and high (n=25, 20% female, mean age 49.6y ± 11.5) lymphatics based on the median number of lymphatic vessels per area at 1 year. We focused on the 1 year timepoint as this period has the potential to critically influence the trajectory of CAV and has vast therapeutic implications for future studies. Averaged lymphatic area increased in the low lymphatic group between 1 and 5 years (1.7 ± 2.7 and 28.9 ± 53.2 vessels/mm<sup>2</sup>) but remained below the averaged area for the high lymphatic group (79.8 ± 123.5 and 77.5 ± 176.7 vessels/mm<sup>2</sup>).

**Table 6. Recipient characteristics based on lymphatic area.**

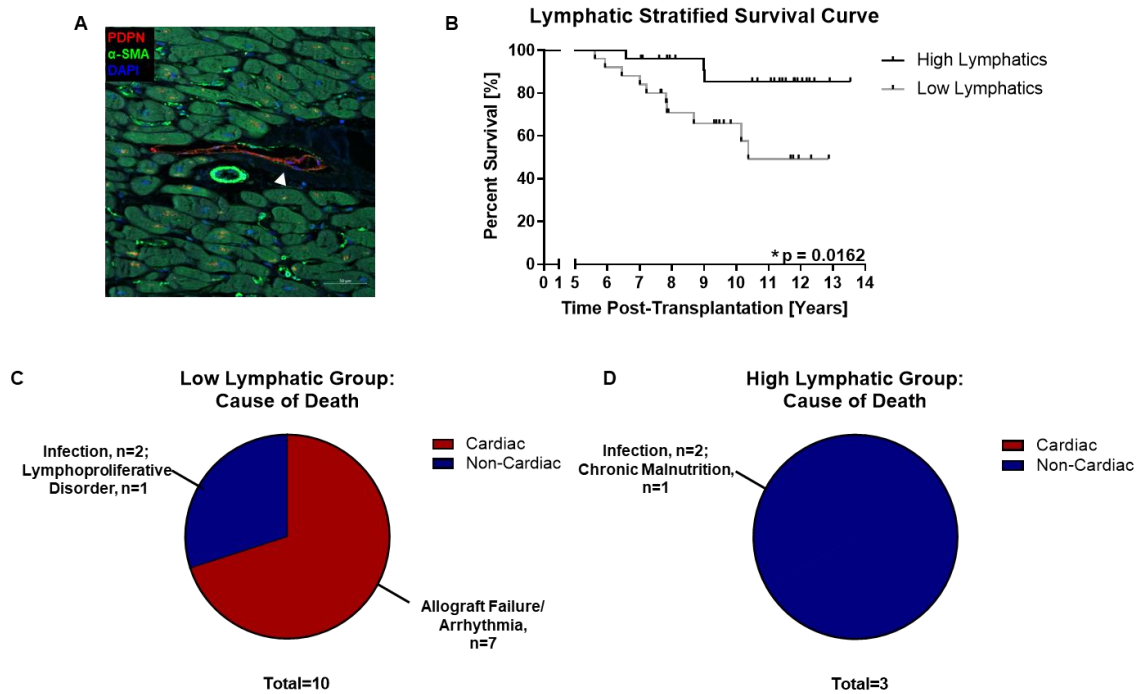
<b>Variable</b>	<b>Low Lymphatics (<math>\leq 7.905</math>, n=25)</b>	<b>High Lymphatics (<math>&gt; 7.905</math>, n=25)</b>	<b>p-value</b>
Recipient Age, y	48.4 $\pm$ 11.0	49.6 $\pm$ 11.5	0.7225
<b>Gender, n (%)</b>			
Male	20 (80%)	20 (80%)	1.0000
Female	5 (20%)	5 (20%)	1.0000
<b>Race, n (%)</b>			
Caucasian	12 (48%)	11 (44%)	0.6767
Black	13 (52%)	14 (56%)	0.7003
<b>HF Etiology, n (%)</b>			
Ischemic Cardiomyopathy	7 (28%)	5 (20%)	0.2482
Non-ischemic Cardiomyopathy	18 (72%)	20 (80%)	0.5164
<b>Medical Risk Factors, n (%)</b>			
Diabetes	12 (48%)	11 (44%)	0.6767
Hypertension	15 (60%)	20 (80%)	0.0910
Kidney Disease	6 (24%)	8 (32%)	0.2850
Cancer	4 (16%)	1 (4%)	<b>0.0073</b>
CMV Mismatch, n (%)	4 (16%)	5 (20%)	0.5050
Ischemic Time, n (%)	217 $\pm$ 54	220 $\pm$ 38	0.7920
<b>Rejection Episodes, #</b>			
1R	11.2 $\pm$ 5.6	10.9 $\pm$ 6.2	0.8329
2R	1.2 $\pm$ 1.7	1.1 $\pm$ 1.4	0.7923

Recipient characteristics of the low and high lymphatic groups were well matched aside from the incidence of pre-existing cancer prior to transplantation (16% vs. 4%,  $p = 0.0073$ ; Table 6). Donor data revealed that the low lymphatic group had lower average ages ( $26y \pm 8$ ) compared to the high lymphatic group ( $28y \pm 8$ ; Table 7). Most notably, the low lymphatic group had more female donors ( $n=2$ ,  $p = 0.0047$ ) where the high lymphatic group had none.

**Table 7. Donor characteristics based on lymphatic area.**

<b>Variable</b>	<b>Low Lymphatics (<math>\leq 7.905</math>, n=23)</b>	<b>High Lymphatics (<math>&gt; 7.905</math>, n=25)</b>	<b>p-value</b>
Donor Age, y	26 $\pm$ 8	28 $\pm$ 8	0.3588
<b>Gender, n (%)</b>			
Male	23 (92%)	25 (100%)	0.5637
Female	2 (8%)	0 (0%)	<b>0.0047</b>
Gender-matched	20 (80%)	20 (80%)	1.0000
Gender-mismatched	5 (20%)	5 (20%)	1.0000
<b>Cause of Death, n (%)</b>			
Anoxia (Drug Intoxication)	1 (4%)	1 (4%)	0.6694
Anoxia (Other)	2 (8%)	3 (12%)	0.2848
Head Trauma	20 (80%)	19 (76%)	0.0563
Cerebrovascular Event	2 (8%)	2 (8%)	0.5459

Next, we investigated if lymphatics at 1 year was associated with long-term survival in these patients. After 10 years post-HTx, mortality was significantly higher in the low vs. high lymphatic group (40% vs. 12%,  $p = 0.0162$ ; Figure 4B). This significant difference in survival did not change even after adjustment for age, gender, and race (cox proportional hazard model,  $p=0.0293$ ). Of the 10 patients that died in the low lymphatic group, 7 died from complications of allograft dysfunction (70%, Figure 4C) and 7 had evidence of CAV near time of death (CAV<sub>2</sub>, n=1; CAV<sub>3</sub>, n=6). None of the deaths in the high lymphatic group were from a cardiac-related causes (sepsis, n=2; chronic malnutrition, n=1; Figure 4D) but 2 had evidence of CAV at their time of death (CAV<sub>1</sub>, n=1; CAV<sub>3</sub>, n=1). These data suggest that cardiac lymphatics are important to allograft function and survival.



**Figure 4. Cardiac lymphatics predict survival after heart transplantation.** Immunohistochemistry utilized podoplanin (PDPN, arrow, red) to identify lymphatic vasculature and alpha-smooth muscle actin ( $\alpha$ -SMA, green) to identify blood vasculature on endomyocardial biopsy samples (A, 20x). Patients with fewer cardiac lymphatic vessels on endomyocardial biopsies at 1 year had reduced survival (B). Patients with lower lymphatics had more cardiac related causes of death (C) compared to patients with higher lymphatics (D). Kaplan-Meier curve utilized log-rank testing. Differences of  $p < 0.05$  were considered significant.

### 3.4.3 Immunosuppression

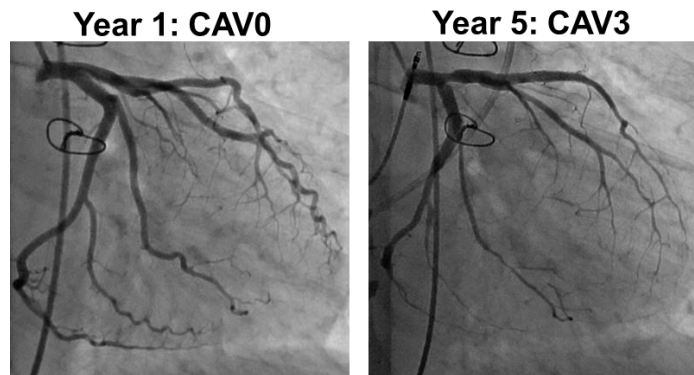
All patients were administered different combinations of ISDs including but not limited to CNIs, mTOR inhibitors/antiproliferative agents, and steroids (Table 8). Tacrolimus was utilized to replace cyclosporine in the early 2000s and therefore is only present in patients that received transplants around that time. Both low and high lymphatic groups had similar ISD regimens 1 year after HTx where tacrolimus (84% vs. 96%,  $p = 0.3711$ ), MMF (80% vs. 92%,  $p = 0.3602$ ), and prednisone (64% vs. 64%,  $p = 1.0000$ ) dominated. Treatment decisions were made at the discretion of the practicing physician but

tended to include sirolimus when CAV was suspected. Patients with lower lymphatic amounts had marginally higher percentages of sirolimus in their treatment regimen at 5 years compared to the high lymphatic group albeit not significant (48% vs. 36%,  $p = 0.1904$ ). Sirolimus is known to inhibit the growth and proliferation of lymphatic cells and is used to treat lymphatic malformations and lymphoproliferative disorders.<sup>152</sup> Since sirolimus becomes more prevalent as CAV severity increases, this population is at higher risk for long-term lymphatic inhibition.

**Table 8. Immunosuppressive drug use stratified by lymphatics.**

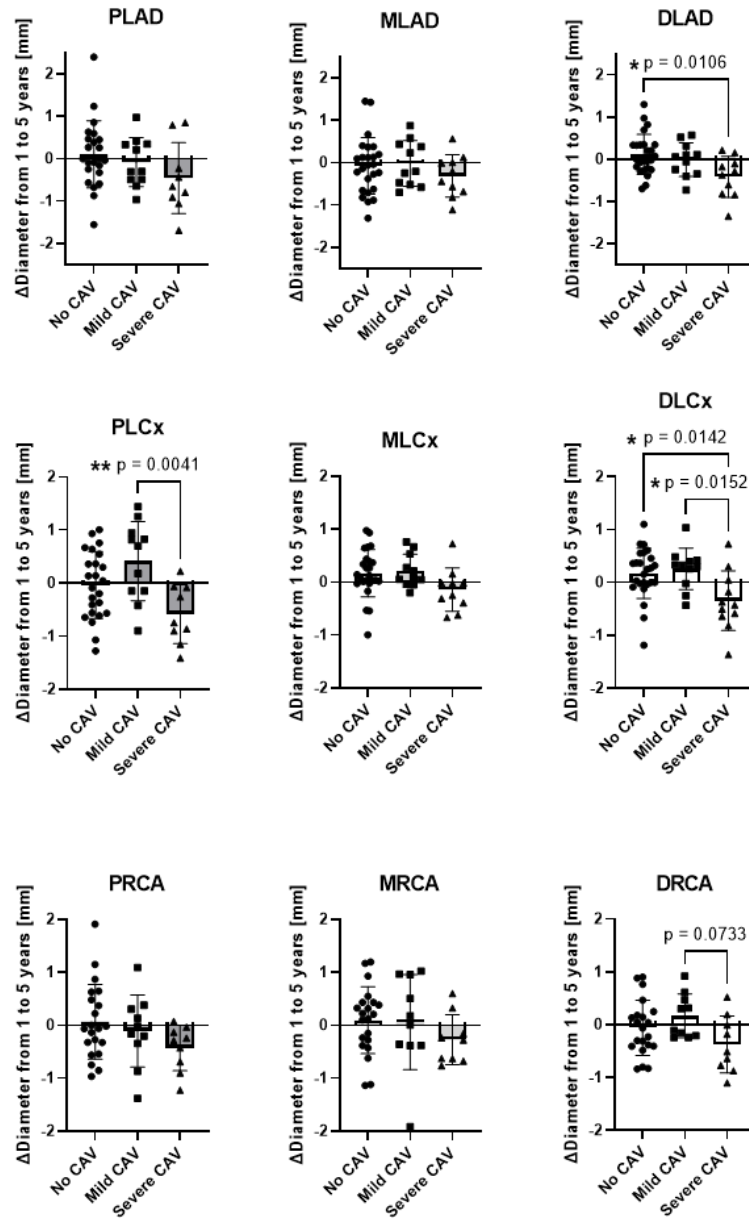
<b>Immunosuppressive Regimen</b>	<b>Low Lymphatics (<math>\leq 7.905</math>, n=25)</b>	<b>High Lymphatics (<math>&gt; 7.905</math>, n=25)</b>	<b>p-value</b>
<b>1 Year, n (%)</b>			
Tacrolimus	21 (84%)	24 (96%)	0.3711
Sirolimus	2 (8%)	1 (4%)	0.2482
Mycophenolate	20 (80%)	23 (92%)	0.3602
Prednisone	16 (64%)	16 (64%)	1.0000
Cyclosporine	2 (8%)	0 (0%)	<b>0.0047</b>
<b>5 Years, n (%)</b>			
Tacrolimus	18 (72%)	22 (88%)	0.2059
Sirolimus	12 (48%)	9 (36%)	0.1904
Mycophenolate	15 (60%)	18 (72%)	0.2963
Prednisone	9 (36%)	8 (32%)	0.6276
Cyclosporine	1 (4%)	0 (0%)	<b>0.0455</b>

#### 3.4.4 Angiogram analysis



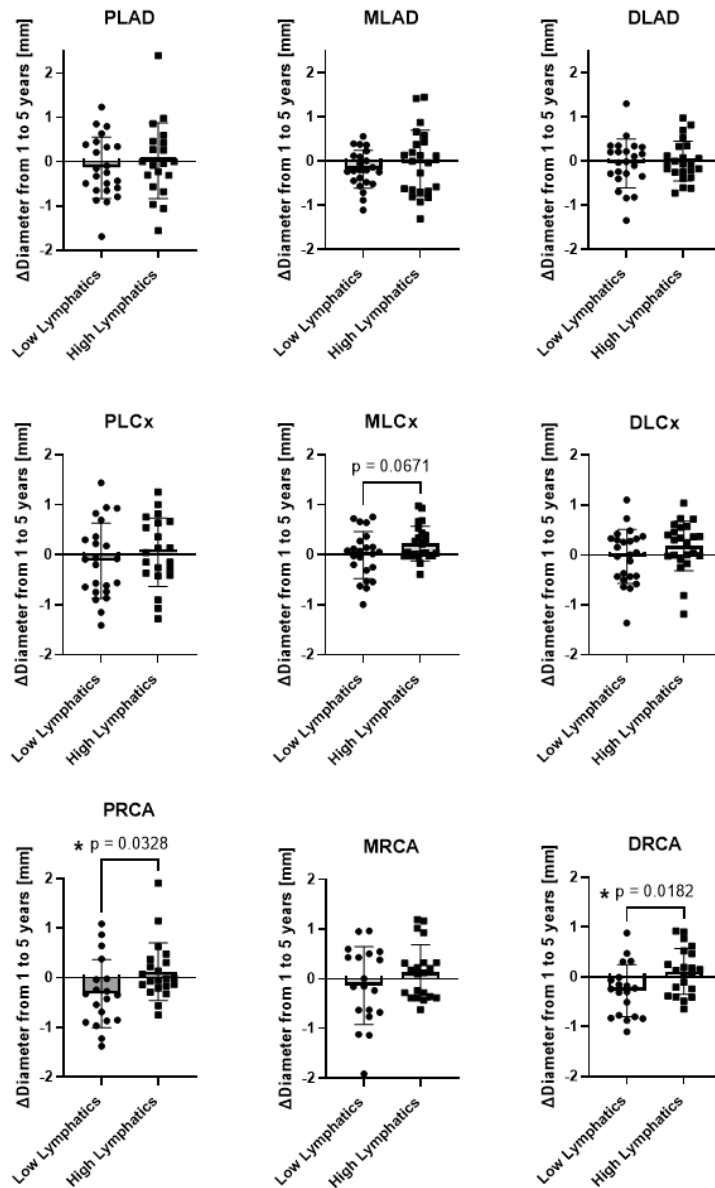
**Figure 5. Images depict CAV progression from 1 to 5 years in the same patient and angiographic view used for analysis.**

Finally, we sought to utilize this cohort to test if lymphatics correlated to coronary narrowing indicative of CAV. The unbiased, blinded angiographic analysis presented within this study intended to determine more quantifiable metrics to link luminal narrowing present in CAV patients to individual lymphatic quantities (Figure 5). Distal segments of the LAD, LCx, and RCA in severe CAV<sub>2-3</sub> patients presented significant size decrement compared to the no CAV<sub>0</sub> and/or mild CAV<sub>1</sub> cohorts (LAD,  $p = 0.0106$ ; LCx,  $p = 0.0142$ ,  $p = 0.0152$ ; RCA,  $p = 0.0733$ ; Figure 6), which aligns with the expected progression based on CAV severity. Patient with lower lymphatic quantities observed significant narrowing of the distal RCA segment ( $p = 0.0182$ ) and a trending change in the mid LCx ( $p = 0.0671$ ; Figure 7). Additional segments had similar albeit non-significant trends. While not significant, patients with higher lymphatic quantity tended to have less diameter loss across coronary segments.



**Figure 6. Angiographic diameter measurements revealed significant vessel narrowing in distal segments when stratified by disease severity.** Landmarks used for segmental analysis to enhance specificity of measurements based on anatomic locations. Quantification of the diameter change from one to five years post-transplantation stratified by disease severity. Statistical analysis utilized a one-way ANOVA with Bonferroni's post-hoc testing. Differences of  $p < 0.05$  were considered significant. LM = left main coronary artery, LAD = left anterior descending, LCx = left circumflex, RCA = right coronary artery,

D1 = first diagonal branch, OM1 = first obtuse marginal branch, R2 = right ventricular branch, R3 = acute marginal artery, P- = proximal, M- = middle, D- = distal.



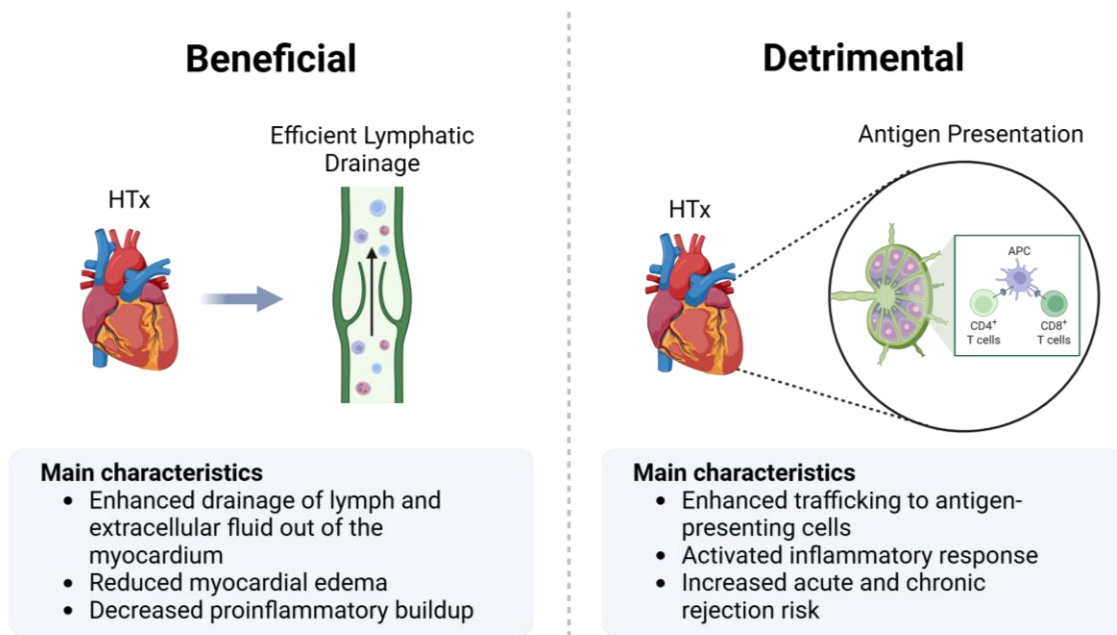
**Figure 7. Lymphatic stratification revealed significant decreases in RCA diameter after angiographic analysis.** Quantification of the diameter change from one to five years post-transplantation stratified by lymphatic area. Statistical analysis utilized a two-way unpaired t-test. Differences of  $p < 0.05$  were considered significant. LM = left main coronary artery, LAD = left anterior descending, LCx = left circumflex, RCA = right

coronary artery, D1 = first diagonal branch, OM1 = first obtuse marginal branch, R2 = right ventricular branch, R3 = acute marginal artery, P- = proximal, M- = middle, D- = distal.

### **3.5 Discussion**

To the best of our knowledge, this is the first study to identify a correlation between reduced cardiac lymphatics and increased late mortality after HTx. Our study builds on a published study showing that short-term decreases in a lymphatic marker correlated with worse rejection episodes in EMBs taken 0.5 months after HTx.<sup>153</sup> While it has been shown that renal transplant biopsies with higher lymphatic densities were associated with improved functional outcomes<sup>97</sup>, contrasting animal studies have shown inhibiting lymphatics after heterotopic HTx diminished cardiac inflammation extending graft survival<sup>97,99</sup>. However, animal modeling in other cardiovascular disease states like MI have proven pro-lymphangiogenic therapies that promote lymphatic regeneration after injury improve cardiac functional outcomes by ameliorating edema and inflammatory stagnation.<sup>19,21,129,154</sup> This dichotomy in part could be due to organ-, species-, or time-dependent differences but warrants further investigation on lymphatics as a novel therapeutic target to treat or forestall rejection (Figure 8).

# Lymphangiogenesis after Heart Transplantation



**Figure 8. Schematic depicting the duality of lymphatics in heart transplantation.**

Cox proportional hazard modeling was implemented to adjust for age, gender, and/or race differences within our population. Even though our finding was still significant after adjustment for these variables it is important to discuss how age, gender, and race can affect transplant outcomes and lymphatic regeneration. Age is a complex factor in long-term survival after HTx. On one hand, higher recipient and donor ages increase post-operative complications (i.e. PGD), comorbidities (i.e. diabetes, hypertension, chronic kidney disease, etc.), and age-related functional cardiovascular decline.<sup>155,156</sup> On the other hand, younger recipients are also known for worse transplant outcomes despite having better overall health.<sup>157</sup> This can be attributed to younger individuals having higher risk behaviors affecting adherence, a more robust immune response leading to higher rejection rates, and a higher risk for transplant vasculopathy complicating transplant outcomes.<sup>157,158</sup> Our data reveals the lowest average ages lie in both the low lymphatic and severe CAV<sub>2-3</sub>

groups. This trend would be interesting to follow in an expanded cohort but is limited by the small number of younger patients transplanted at Emory University. Gender of the recipient and donor can introduce variations in biological responses, risk factor profiles, and psychosocial factors. Estrogen production can be cardioprotective by maintaining lower blood pressure through nitric oxide production, improving lipid profiles preventing atherosclerosis or other cardiovascular diseases, reducing oxidative stress on endothelial cells, etc.<sup>159,160</sup> These factors innately decrease the risk profiles of cardiovascular disease on pre-menopausal females and explain why the HTx community is predominantly composed of males. Nevertheless, females that undergo HTx tend to have stronger immune responses that lead to higher rates of acute rejection.<sup>161,162</sup> This has been previously linked to estrogen's protective effects on lymphatic health by inducing lymphangiogenesis and increasing drainage.<sup>163</sup> Moreover, psychosocial factors are important for recovery and adherence to medication regimens. Females are often reported to have higher levels of social support that improve long-term adherence and mental health thereby positively influencing transplant outcomes.<sup>164</sup> Interestingly, the only two donor females within this cohort were in both the low lymphatic and severe CAV<sub>2-3</sub> groups. Even with the beneficial factors listed above, female donors and recipients tend to have worse outcomes post-HTx and higher risk of transplant vasculopathy which is reflected specifically in the donor females within our study. For female recipients this trend is explained in part by older recipient ages at the time of surgery increasing the risk of complications and comorbidities. Identifying pre- and post-menopausal gender-based differences in lymphatic structure and function post-transplantation could provide new insight into longitudinal rejection risk assessments. While race was similar in both our disease- and lymphatic-based recipient

cohorts, it has been noted that Black recipients tend to have higher post-HTx complications and mortality compared to Caucasian counterparts.<sup>165</sup> Race itself does not affect lymphatic health but disparities in socioeconomic barriers affecting access to care and genetic factors influencing the immune response or comorbidities can lead to significant differences in lymphatic function similar to transplant outcomes. While improving, transplantation and lymphatic research has a historical underrepresentation of racial minorities. Adjusting our perspective on this study to focus on age, gender, or race could lead to novel and important information targeted towards closing the existing research gap.

**Table 9. Immunosuppressive drug use stratified by disease severity.**

Immunosuppressive Regimen	Overall (n=50)		Severe CAV <sub>2-3</sub> (n=12)		Mild CAV <sub>1</sub> (n=12)		No CAV <sub>0</sub> (n=26)	
	1 Year	5 Year	1 Year	5 Year	1 Year	5 Year	1 Year	5 Year
Tacrolimus	45 (90%)	40(80%)	12 (100%)	9 (75%)	10 (83%)	9 (75%)	23 (88%)	22 (85%)
Sirolimus	3 (6%)	21 (42%)	1 (8%)	8 (67%)	0 (0%)	6 (50%)	2 (8%)	7 (27%)
Mycophenolate	43 (86%)	33 (66%)	9 (75%)	6 (50%)	10 (83%)	6 (50%)	24 (92%)	21 (81%)
Prednisone	32 (64%)	17 (34%)	11 (92%)	9 (75%)	6 (50%)	2 (17%)	15 (58%)	6 (23%)
Cyclosporine	2 (4%)	1 (2%)	0 (0%)	0 (0%)	1 (8%)	1 (8%)	1 (4%)	0 (0%)

Although there are no randomized controlled trials, smaller trials suggest sirolimus is beneficial at preventing progression of CAV by inhibiting mTOR. mTOR inhibition blocks the activation and proliferation of T- and B-cells which are integral in the rejection process. As expected, sirolimus is highly prevalent in our severe CAV<sub>2-3</sub> cohort at 5 years (75%, Table 9). In addition to sirolimus stunting the immune response, it also has the ability to inhibit lymphatic proliferation and is commonly used to treat lymphatic malformations. The anti-lymphangiogenic effects of sirolimus are well documented in hyperproliferative pathologies but whether this ISD would have undesirable affects in the context of

transplantation is uncertain. Our data shows the lower lymphatic group experienced a higher percentage of sirolimus than the higher lymphatic group at 5 years albeit insignificant. This finding suggests ISD could present a potential confounding factor in our data as it is unknown the degree to which sirolimus affects cardiac lymphatics post-HTx. Future analysis should adjust for sirolimus use and further investigation of this mTOR inhibitor's effect on cardiac lymphatics in HTx patients should be conducted. Moreover, including additional centers and expanding our patient cohort will diversify ISD regimens within the patient population and reduced any single-center bias.

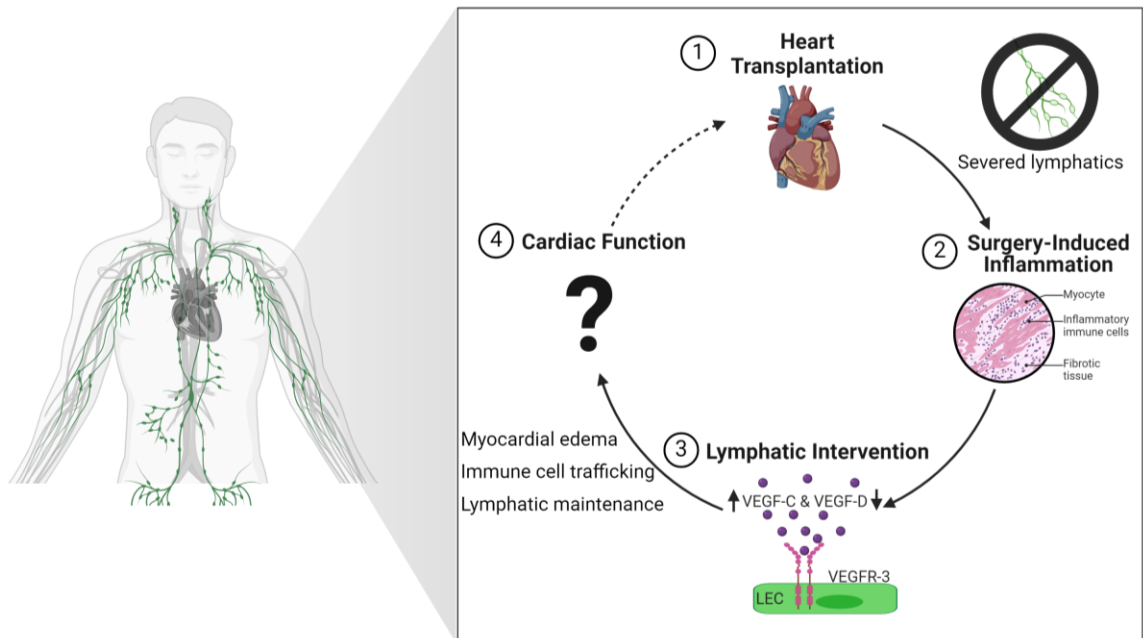
Angiography is the clinical norm for identifying transplant vasculopathy but has many limitations. While angiograms are excellent at visualizing arterial blockages, they may miss the more subtle changes associated with transplant vasculopathy. The treating physician qualitatively visualizes coronary narrowing to determine CAV severity. This alone introduces bias and limits the detection of early or mild CAV cases. Our goal in analysing the diameter change over time for these patients was to establish a more quantitative approach to identifying coronary health and transplant vasculopathy with readily available imaging. Increases in disease severity showed expected trends in distal narrowing over time but there were minimal significant differences when stratified by lymphatic area. This result could be in part due to the two-dimensional nature of the analysis. Ideally, a volumetric-based quantification strategy would be utilized to be accurately assess this three-dimensional disease. Additional imaging techniques like intravascular ultrasound (IVUS) or optical coherence tomography (OCT) could lead to more functional assessments of coronary health but are not uniformly conducted across transplant centers like angiography. IVUS and OCT imaging would allow high-resolution,

cross-sectional images of the coronaries to be analysed for more extensive vessel parameters like intimal hyperplasia, volumetric dimensional changes, and plaque volume/composition. To get a more comprehensive view on transplant vasculopathy within our patient cohorts, it will be imperative to incorporate a combination of angiography and other imaging techniques when available.

Understanding the longitudinal structural changes of the lymphatic vasculature after heart transplantation is crucial to how it influences graft survival and function. This study is limited to assessing the lymphatic vasculature found within the EMBs and may not necessarily reflect cardiac lymphatics throughout the entirety of the heart. Animal modeling will be essential to demonstrating how altered lymphatic drainage affects transplant vasculopathy by giving incremental incite on structural adaptations, local inflammation, and drainage functionality. This characterization could be the steppingstone to creating novel therapeutics for transplant rejection and CAV. Meanwhile, modification of surgical techniques and immune suppression may be possible to better preserve extracardiac lymphatic connections or promote their restoration. Future studies should focus on understanding the mechanism by which lymphatics influence allograft survival from both an immunomodulatory and a drainage standpoint. Although this study is limited by small size, single-center, and retrospective nature, the strong association shown between lymphatics and survival should generate new avenues of research to improve outcomes and long-term survival after HTx.

### 3.6 Conclusions

The results of this study have direct implications for HTx survival by identifying a novel marker associated with increased mortality. Our study was able to incorporate diverse clinical metrics to shed light on lymphatic differences within our patient population. Upon further validation, this lymphatic quantification strategy could be used in patients to identify those at higher risk for poorer outcomes or offer a novel metric to assess the health of organs prior to procurement. As a whole, this study provides a foundation for lymphatic research in clinical HTx patients and should garner interest as a potential pathway for therapeutic interventions (Figure 9).



**Figure 9. Schematic of suspected lymphatic affects after heart transplantation.**

## CHAPTER 4. LONGITUDINAL EVOLUTION OF CARDIAC LYMPHATICS FOLLOWING HEART TRANSPLANTATION

### 4.1 Abstract

*Introduction:* After heart transplantation, donor lymphatic vasculature remains intact but connections to recipient systemic lymphatics are lost and not surgically re-established. The heart is comprised of an extensive lymphatic network known to contribute to normal cardiac function and healing after injury by enhancing clearance of immune cells and extracellular fluid from the heart. We hypothesized that the altered lymphatic flow may affect allograft function by preventing the egress of proinflammatory cells and interstitial fluid out of the myocardium. *Methods & Results:* Lymphatic remodeling was studied in a rodent model of heterotopic abdominal heart transplantation. Transplanted hearts had increased total podoplanin+ (PDPN) lymphatic vessels at all timepoints but only significantly at day 14 and day 28 compared to native heart counterparts. These transplants had significant enlargement of PDPN+ lymphatic structures indicating potential drainage deficits with stagnant lymph reservoirs. Lymphatic drainage of transplanted and non-transplanted control hearts was assessed via intramyocardial injection of dextran-cyanine5.5. Day 14 transplants had the most significant elevation of circulating dextran values at 2 hours compared to both non-transplanted controls and 3 and 7 day transplanted hearts. These data coincide with previously published work identifying day 14 as a critical timepoint in restoration of functional lymphatic drainage after solid-organ transplantation, suggesting physiologic processes important to lymphatic restoration increase with time after transplantation. *Translational Impact:* These data coupled with functional and

immunologic cardiac metrics support lymphatic involvement in the etiologies of cardiac transplantation and provide a foundation for lymphatic-based therapeutic intervention.

## 4.2 Introduction

The lymphatic network regulates immune cell trafficking, tissue-fluid homeostasis, and nutritional lipid uptake and transport.<sup>16,99</sup> Lymphangiogenesis is an essential component of lymphatic development, restoration, and regeneration.<sup>102,111,112</sup> This process plays an unequivocal role in maintaining an effective drainage system for all tissues and organs. The process of lymphangiogenesis is driven through the VEGF family.<sup>17,102</sup> VEGFR-3 is a type of tyrosine kinase receptor that forms homodimers and undergoes autophosphorylation to activate kinase activity when bound to VEGF-C or VEGF-D.<sup>103</sup> VEGF-C/VEGFR-3 signaling is the main pro-lymphangiogenic pathway that induces AKT and ERK activation to regulate lymphatic endothelial cell (LEC) migration, proliferation, and differentiation.<sup>17,99,103</sup> Overexpression of VEGF-C establishes pro-lymphangiogenic effects by providing directional cues for LEC migration and lymphatic vessel augmentation.<sup>104,105</sup> In recent years, pro-lymphangiogenic therapies have proven to reduce fluid retention, improve immune cell clearance, and decrease fibrosis after MI in rodent models.<sup>18-22</sup> Additionally, anti-lymphangiogenic therapies (i.e. MAZ-51) exacerbated disease states by blocking VEGF-C and VEGF-D induced phosphorylation of VEGFR-3.<sup>132,133</sup> These studies suggest administration of lymphatic growth factors or their antagonists provide a potential avenue to target lymphatic vessels in diseases of every vascularized organ.

The heart is comprised of a rich lymphatic network, but very little is known about the physiologic and pathophysiologic role of cardiac lymphatics. The route of cardiac lymphatic drainage flows from subendocardial to subepicardial lymphatics towards the mediastinal lymph nodes.<sup>22</sup> The consequence of having impaired cardiac lymphatic flow may explain many unknown pathologic entities. This gap in knowledge offers a distinctive opportunity to pinpoint the lymphatic system's role in many pathophysiologic conditions, particularly heart transplant rejection. During HTx, the lymphatic collecting vessels are severed at the time of heart excision and not surgically reconnected in the recipient. The consequences resulting from disrupted lymphatic drainage in transplanted hearts remain elusive. In our clinical data, we identified decreases in lymphatic area correlated to increased mortality. This novel finding made us want to dive deeper into characterizing lymphatics after HTx with the plan to eventually introduce lymphangiogenic therapies intended to combat transplant rejection.

Animal modeling enabled us to bridge the gap between laboratory research and clinical application by allowing us to investigate intricate details surrounding graft survival, vasculopathy, and even transplant rejection. HAHT in rodents is an established model utilized to study transplant immunology, graft survival, and the effects of immunosuppressive treatments.<sup>83,166,167</sup> This model provides crucial information on transplant biology and therapeutic interventions. Reported strain differences in baseline inflammatory sensitivities provide a proven model to test the effects of immune activation after HAHT.<sup>93,168,169</sup> Exchanging cardiac allografts across minor histocompatibility barriers of these strains found many pathologic factors (i.e. arteriosclerotic lesions, cellular infiltration, intimal thickening, etc.) identical to outcomes in human cardiac allografts,

while transplantation within a strain has less rejection due to lower antigen and HLA class diversity.<sup>94</sup> Conversely, many limitations exist with the heart being implanted into the abdomen. The transplant has altered circulatory function and hemodynamic interactions that can reduce relevance in studying long-term cardiac functionality or performance. Its abdominal placement also introduces the graft to a different immune microenvironment than the thoracic cavity. This has the potential to alter the recipient's immune response and limits the direct applicability to human grafts. Future studies involving therapeutic intervention will need to include large animal modeling where orthotopic heart transplantation is possible to improve clinical translation.

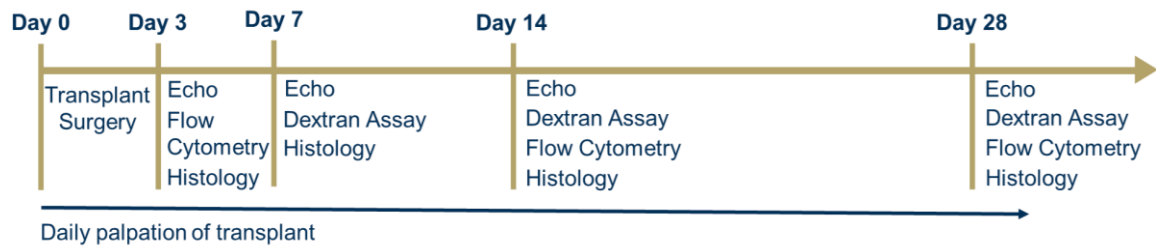
We hypothesized disruption of lymphatic drainage after HTx deteriorates cardiac functionality by impeding the egress of interstitial fluid and pro-inflammatory infiltrates out of the myocardium exacerbating transplant outcomes. This study is designed to characterize lymphatic changes in quantity, structure, and function longitudinally after HAHT in antigen-matched animals. Only then can we begin to correlate lymphatics to pathologic factors of transplant rejection utilizing an antigen-mismatched model. Eventually, our goal is to utilize lymphatics as a therapeutic target intended to improve graft function and extend survival.

### **4.3 Methods**

Animals were handled in accordance to the *Guide for the Care and Use of Laboratory Animals* instituted by the National Institutes of Health (NIH). Study protocols are approved by Emory University's Institutional Animal Care and Use Committees.

### 4.3.1 Heterotopic abdominal heart transplant (HAHT)

Adult rats approximately 8 weeks of age (250g body weight) were purchased from Charles River. Animals underwent standard housing conditions with a normal light cycle. Animal experiments were conducted in accordance with the Emory University's Institutional Animal Care and Use Committee (IACUC). Experimental study design is outlined in Figure 10. Anesthesia was induced by isoflurane inhalation at 1-3% and maintained at 1.5-2% with a flow rate of 0.5 liters per minute. Analgesic regimens for the recipient consisted of 1 mg/mL Buprenorphine ER (1 mg/kg; Wedgewood) at the start and 2.5mg/mL Bupivacaine (4 mg/kg; McKesson) at the end of surgery.



**Figure 10. Study design for HAHT in antigen-matched animals.**

#### 4.3.1.1 Recipient Preparation

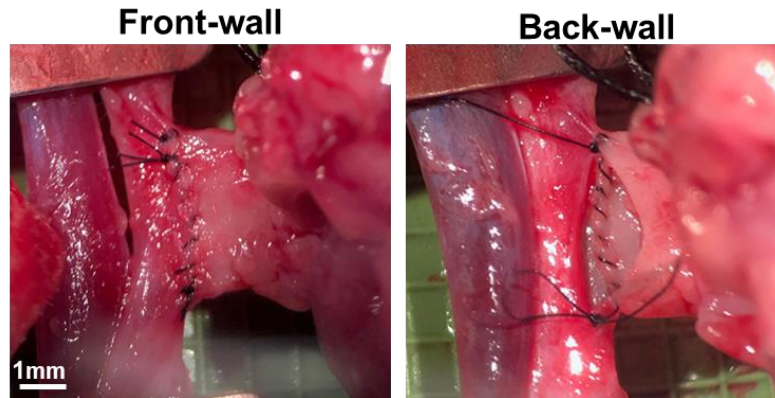
Animal was placed in a supine position and a longitudinal incision was made in the center of the abdomen. Abdominal contents were gently retracted to expose the abdominal aorta (aAo) and inferior vena cava (aIVC). A rubber background was threaded underneath the aAo and aIVC to protect the spine and spinal nerves.

#### 4.3.1.2 Donor operation

Animals underwent a bilateral thoracotomy that allowed the anterior chest wall to be lifted cranially. Approximately 0.5 mL of heparin (1,000 USP/mL, McKesson) was injected into the thoracic inferior vena cava (tIVC). 5-0 silk sutures (Ethicon, 1925G) were utilized to ligate the tIVC as well as the left and right superior vena cava (SVC). The thoracic ascending Aorta (tAo) was severed with scissors just distal to the brachiocephalic branch, while the pulmonary artery (PA) was severed distal to its bifurcation to left and right segments. A 5-0 silk suture was placed around the base of the heart to successfully ligate the pulmonary trunk. The donor heart was carefully excised and dissected from any connective tissue. The tAo was flushed with 0.5 mL of heparin and immediately placed in ice-cold saline.

#### 4.3.1.3 Recipient operation

Dual micro-clamp (Fine Science Tools, 18040-22) was utilized to interrupt blood flow distal to the renal arteries but proximal to the aortic bifurcation. While gently lifting the anterior wall of the aAo and aIVC with micro-forceps, micro-scissors (WPII, 14003) were able to make a 2-4mm longitudinal incision leaving the posterior wall undisturbed. The elliptical openings of the aAo and aIVC were flushed with saline to remove any residual blood. An end-to-side anastomosis of the donor tAo to the recipient aAo and the donor PA to the recipient aIVC was performed with 8-0 nylon sutures (Fine Science Tools, 12051-08) in left and right continuous segments (Figure 11). Dual micro-clamp was released and heart was observed for return normal sinus rhythm prior to close.



**Figure 11. Surgical images of front- and back-wall of the aortic anastomosis.** The aIVC is shown on the left and the aAo is oriented on the right.

#### 4.3.2 *Echocardiography*

VEVO 3100 In Vivo Micro-Imaging System (FUJIFILM VevoSonics) was utilized to confirm transplant viability. Simultaneous echocardiography and electrocardiography were performed on the transplanted heart in the abdominal cavity at the time of sacrifice. Anesthesia was induced using 1-3% isoflurane and maintained at 1-1.5%. Concurrent clips of the transplanted heart were taken in B- and M-mode utilizing a 15-30 Hz ultra-high frequency linear array transducer (VisualSonics, MX250). Clips were attempted at 135 frames per second in the parasternal long axis view but were dependent on transplant positioning within the abdominal cavity. All analysis was conducted in Vevo Lab software.

#### 4.3.3 *Histology and immunohistochemistry*

Samples were formalin-fixed, embedded in paraffin, and cut into 5  $\mu\text{m}$  sections. Slides were stored at room temperature prior to staining. Serial sections were utilized for consecutive staining methods.

#### 4.3.3.1 Immunofluorescence staining

Slides were deparaffinized in xylene for 10 minutes (2x), 100% ethanol for 10 minutes (2x), 95% ethanol for 10 minutes, 70% ethanol for 7 minutes, and 50% ethanol for 7 minutes. Slides were washed in 1x PBS for 5 minutes and permeabilized in 0.1% Triton-X100 buffer for 10 minutes. Wash steps were repeated in 1x PBS for 5 minutes (2x). A hydrophobic barrier pen outlined the target tissue to minimize reagent waste. For antigen retrieval, 10 ug/mL proteinaseK was diluted in 1x PBS and applied to the tissue for 30 minutes followed by two wash cycles in 1x PBS for 5 minutes. Slides were blocked in 10% goat serum diluted with 1x PBS at room temperature for 1 hour. Primary antibody incubation was performed overnight at 4°C in 2% goat serum diluted with 1x PBS. Primary antibodies included PDPN (1:200, Sigma, P-1995) and  $\alpha$ -SMA (1:500, Abcam, ab7817). Wash steps were repeated with 1x PBS for 5 mins (5x). Secondary antibody incubation included goat anti-mouse Alexa Fluor 568 (1:1000, ThermoFisher, A11004) and goat anti-rabbit Alexa Fluor 488 (1:2000, ThermoFisher, A11034) in 2% goat serum diluted with 1x PBS at room temperature for 1 hour. Wash steps were repeated with 1x PBS for 5 mins (5x) then tissue was stained with 300  $\mu$ M DAPI (1:1000, ThermoFisher, D21490) for 10 minutes. Slides were washed in 1x PBS for 5 minutes and mounted in diamond anti-fade medium (ThermoFisher, P36966). Fluorescent images were taken on either the Keyence BZ-X800 widefield or the Zeiss LSM800 confocal microscopes. Quantification of lymphatic and blood vessels was accomplished via ImageJ (National Institutes of Health).

#### 4.3.3.2 Hematoxylin and eosin (H&E) staining

Slides were deparaffinized in xylene for 10 minutes (2x), 100% ethanol for 10 minutes (2x), 95% ethanol for 10 minutes, 70% ethanol for 7 minutes, and 50% ethanol for 7 minutes. Slides were washed in diH<sub>2</sub>O and then placed in hematoxylin (Richard-Allan Scientific, 72604) for 5 minutes. Excess stain was rinsed in running tap water until clear then dipped into diH<sub>2</sub>O. Slides were dipped in acid alcohol 3-4 times, washed in diH<sub>2</sub>O, placed in Scott's solution for 1 minute, and washed again. Primed in 70% ethanol for 1 min, slides were then placed in eosin (Richard-Allan Scientific, 71204) for 1 minute prior to dehydrating the slides through graded ethanol and xylene. Slides were mounted with cytooseal (Richard-Allan Scientific, 8310-4) and stored at room temperature. All imaging occurred on the NanoZoomer (Hamamatsu Photonics), while quantification of infiltrating nuclei was conducted via ImageJ (National Institutes of Health).

#### 4.3.3.3 Masson's trichrome staining

Slides were deparaffinized in xylene for 10 minutes (2x), 100% ethanol for 10 minutes (2x), 95% ethanol for 10 minutes, 70% ethanol for 7 minutes, 50% ethanol for 7 minutes, and dipped in diH<sub>2</sub>O. Slides were then fixed in Bouin's Solution (Sigma, HT10132) at room temperature overnight. Excess Bouin's was rinsed in running tap water then dipped in diH<sub>2</sub>O prior to being stained with Weigert's Iron Hematoxylin solution (Sigma, HT1079) for 5 minutes. Slides were washed, stained in Biebrich Scarlet-Acid Fuchsin (Sigma, HT151) for 5 minutes, and washed again. Slides were set in Phosphotungstic/Phosphomolybdic Acid Solution (Sigma, HT152 and HT153), washed, then stained with Aniline Blue Solution (Sigma, B8563) for 4 minutes. Wash steps were

repeated followed by placing slides in 1% Acetic Acid for 2 minutes and dehydrating through graded ethanol and xylene. Slides were mounted using cyto seal (Richard-Allan Scientific, 8310-4) and imaged on the NanoZoomer (Hamamatsu Photonics). Percent fibrosis was quantified through color deconvolution and thresholding within ImageJ (National Institutes of Health).

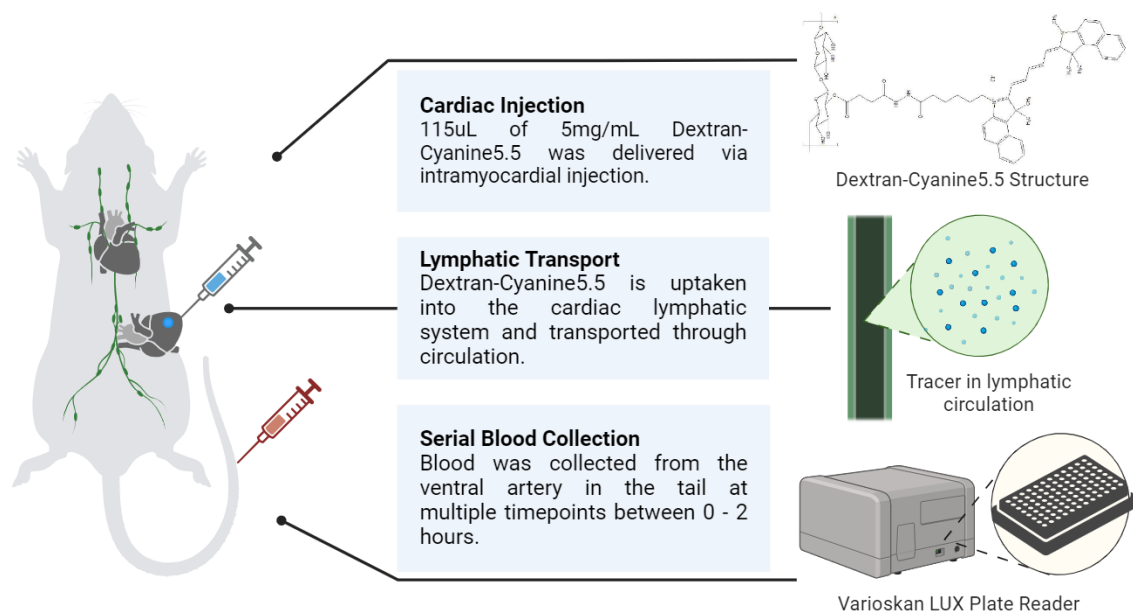
#### 4.3.3.4 Russel Movat's pentachrome staining

Slides were deparaffinized and hydrated to diH<sub>2</sub>O. The following reagents were obtained from a standard kit (Abcam, ab245884). Tissue was stained with working Elastin Stain Solution for 20 minutes, washed in diH<sub>2</sub>O to remove excess stain, then dipped in 2% Ferric Chloride until slides were differentiated appropriately. Differentiation was confirmed microscopically and slides were rinsed in diH<sub>2</sub>O. Slides were placed in 5% Sodium Thiosulfate Solution for 1 minute and rinsed in diH<sub>2</sub>O. 1% Acetic Acid Solution for 2 minutes was utilized to equilibrate the tissue prior to staining with Alcian Blue Solution for 25 minutes. Wash steps in diH<sub>2</sub>O were repeated and slides were placed in Biebrich Scarlet-Acid Fuchsin for 2 minutes. Excess Biebrich Scarlet-Acid Fuchsin was rinsed in diH<sub>2</sub>O then slides were set in 1% Acetic Acid Solution for 5-10 seconds with agitation. Slides were rinsed in diH<sub>2</sub>O and differentiated with 2 changes of 5% Phosphotungstic Acid Solution for 7 minutes each. Proper differentiation was confirmed microscopically prior to rinsing in diH<sub>2</sub>O. Slides were placed in 1% Acetic Acid Solution for 1 minute to remove residual Phosphotungstic acid bound to the tissue and then directly stained with Yellow Stain Solution for 15 minutes. Slides were rinsed in 3 changes of absolute alcohol and then mounted with cyto seal (Richard-Allan Scientific, 8310-4).

Coronary vessel diameters, areas, and thicknesses were quantified utilizing ImageJ (National Institutes of Health).

#### 4.3.4 *Dextran Assay*

We developed a novel dextran assay that evaluated lymphatic drainage by an injection of a 5 mg/mL 500 kDa Cy5.5 conjugated dextran (Creative Biolabs, NTA-2011-ZP118; Figure 12). The dextran was sized at 500 kDa to reduce drainage into regular circulation and be preferentially up taken by the lymphatic vasculature. 115  $\mu$ L of the dextran was loaded into an insulin needle and intramyocardially injected into the LV. The injection volume accounted for the viable space in a rat myocardium that usually ranges from 100  $\mu$ L to 300  $\mu$ L. 115  $\mu$ L was selected as it was important the injection volume could be delivered efficiently without compromising cardiac function or damaging the heart tissue. This injection inevitably increased the interstitial fluid pressure inducing lymphatic uptake and transport through the lymphatics to be dumped back into the circulatory system. Whole blood was collected from the ventral artery of the tail at 0 min, 10 min, 30 min, 1 hr, and 2 hr timepoints. 20  $\mu$ L of whole blood was placed in a black-walled low volume 384-well plate (Corning, 3540), while fluorescent intensity readouts with a 663/690 excitation/emission spectrum were performed by a Varioskan LUX plate reader. Values are interpolated from a standard curve. The assay determined the functional capabilities of the lymphatics within the transplanted heart and is intended linked fluctuations of the immune response and cardiac function.



**Figure 12. Schematic of dextran assay used to estimate lymphatic transport capabilities of the transplanted heart at sacrifice.**

#### 4.3.5 Statistical analyses

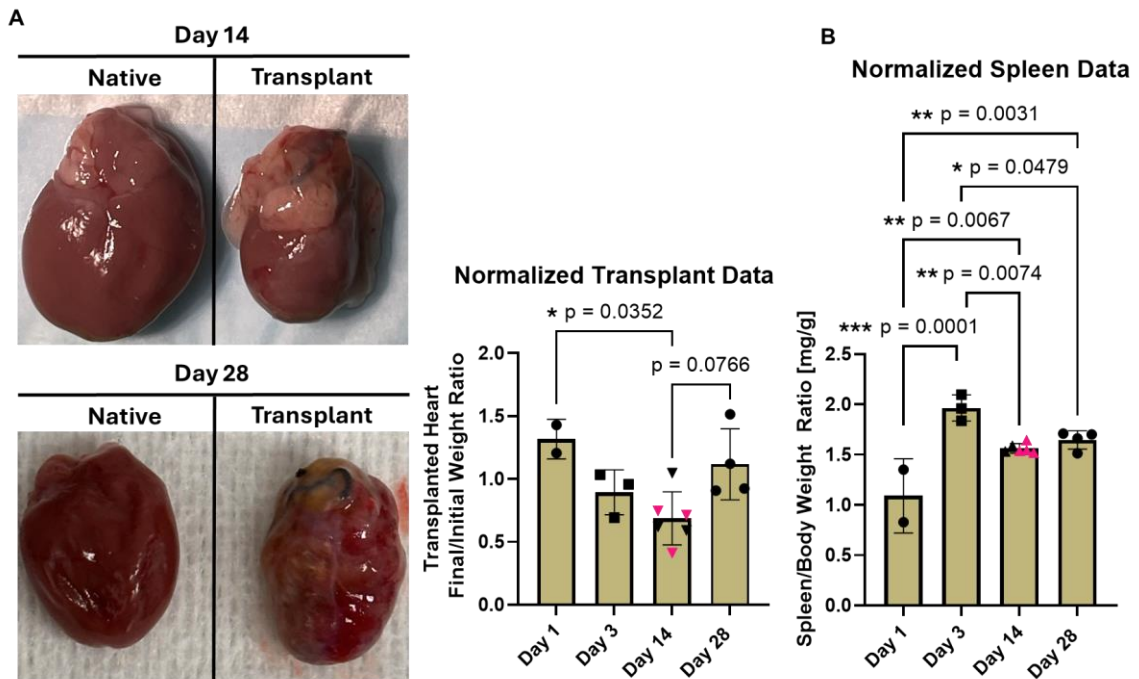
Statistical analysis was performed on GraphPad Prism 9. All tissue was stained and quantified in a blinded fashion. Values were expressed as mean  $\pm$  standard deviation and analysed with either an unpaired t-test, paired t-test, or one-way ANOVA. Any one-way ANOVAs were followed by Tukey's post-hoc testing. All statistical testing was two-sided where p-values of 0.05 or less were considered significant.

## 4.4 Results

### 4.4.1 Fluctuations in heart and spleen size are indicative of graft health

Multiple organs were extracted to observe changes in weight, size and pathological remodeling over time. Transplanted hearts displayed readily identifiable stepwise size and

weight decrements until day 14 ( $p = 0.0352$ ; Figure 13A). Interestingly, this trend did not continue out to day 28 and instead increased ( $p = 0.0766$ ). Another key organ we obtained was the spleen. Spleen weight is commonly used as a biomarker in a variety of physiologic and pathophysiologic conditions as it contributes to processes of the immune response, haematopoiesis, and filtration of the blood. Increased spleen weight is characteristic of splenomegaly and due to an enhanced immune response or chronic inflammation. Our data showed a significant elevation in normalized spleen weights from day 1 to all other timepoints after HAHT (Day 3,  $p = 0.0001$ ; Day 14,  $p = 0.0067$ ; Day 28,  $p = 0.0031$ ), suggesting an increased demand for immune cell production and antigen clearance (Figure 13B).



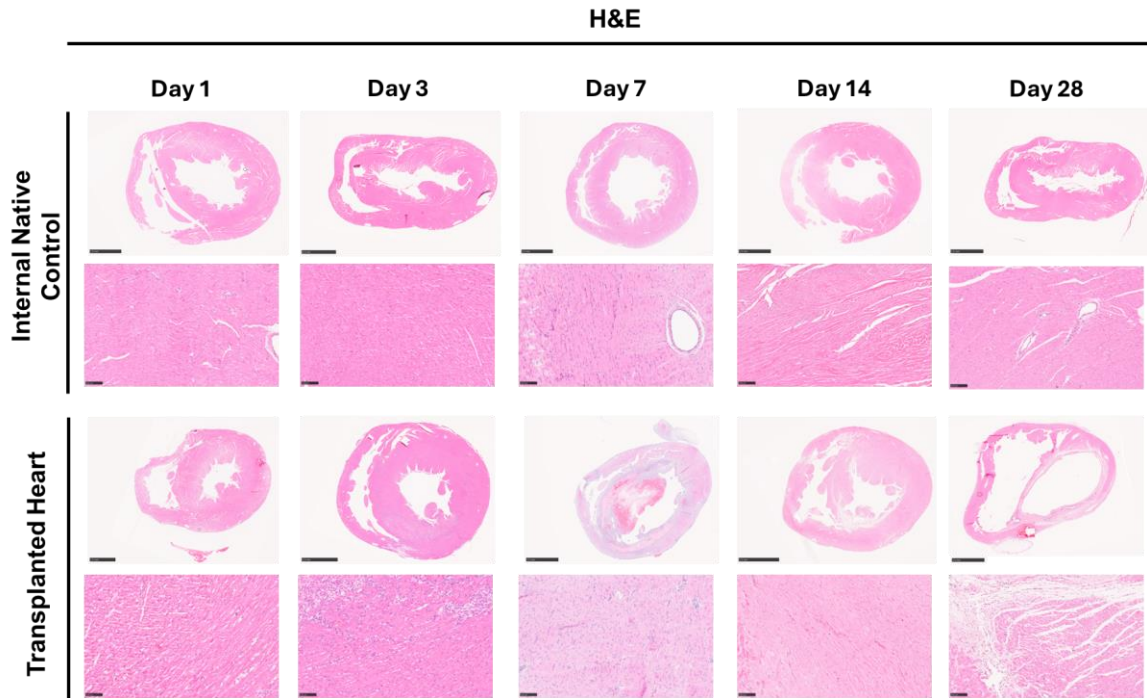
**Figure 13. Fluctuations of heart (A) and spleen (B) size are indicative of graft health and pathologic remodeling.** Black data points represent antigen-matched animals and pink data points represent antigen-mismatched animals. The statistical analysis performed

was a one-way ANOVA with Tukey's post-hoc testing. Error bars indicate SD. \*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$ .

#### 4.4.2 *Unloaded HAHT model instituted morphological and immunological changes in the transplanted heart over time*

Given the unloaded nature of HAHT, we sought to characterize fundamental physical changes in the heart tissue with antigen-matched transplants out to day 28. H&E staining provided rudimentary insight into the structure and morphology of the native and transplanted heart tissue (Figure 14). Transplanted hearts exhibited irregularities in muscle fiber arrangement stained by eosin shown in pink compared to their internal native controls. Deviations from normal muscle fiber structural arrangements suggest extensive ischemic damage to the tissue that led to scar formation, especially at later timepoints. Transplanted hearts that were declining functionally had an increased presence of scar formation and diffuse inflammatory pockets frequently located in the subendocardial regions. Moreover, the interventricular septum was a common area for architectural abnormalities. These areas are more prone to ischemia under conditions of low coronary perfusion pressure as seen in this unloaded HAHT model. Hematoxylin, shown in dark purple, identified inflammatory cells like neutrophils, lymphocytes, and macrophages. Transplanted hearts qualitatively displayed more inflammatory infiltrate that was enhanced in regions of structural remodeling and scar formation. Interestingly, transplanted hearts at day 28 presented with readily visible wall thinning and dilation of the ventricles compared to transplanted hearts at other timepoints. These pathological changes can result from prolonged damage to the heart muscle from conditions like MI, chronic hypertension, or ischemic heart disease.<sup>170</sup> Similar observations in a clinical setting would be attributed to heart failure or

cardiomyopathy. Overall, these data showed that transplanted hearts develop baseline physical characteristics of heart failure as time progresses regardless of the intended disease state or inflammatory microenvironment.

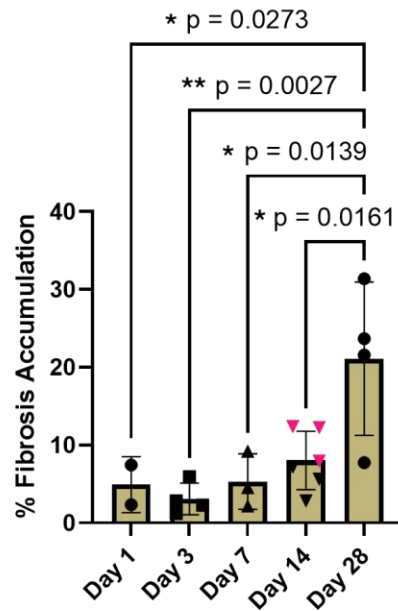
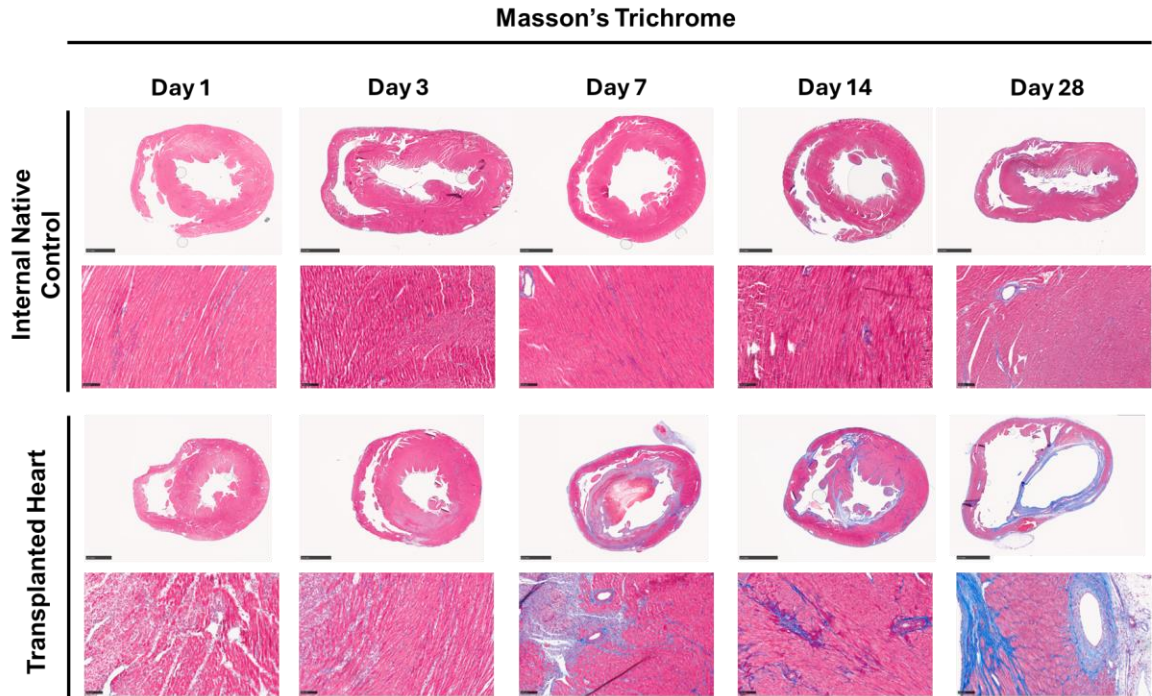


**Figure 14. Unloaded HAHT model induces morphological and immunological changes in the transplanted heart over time.** Enlarged image magnification = 20x, whole-heart image scale bar = 2.5 mm, magnified image scale bar = 100  $\mu$ m.

#### 4.4.3 Fibrosis accumulation increased linearly with time post-HAHT

With the information gained from the H&E images, we wanted to further quantify metrics of fibrosis. Insults to the hearts can cause the myocardial tissue to become injured. When cardiomyocytes cannot repair this injury at a significant enough rate, the heart tends to repair itself by forming fibrous tissue. Masson's Trichrome enabled the identification of collagen via the aniline blue solution (Figure 15). Qualitatively, many transplanted hearts had large sections of the interventricular septum affected by collagen deposition as time

progressed. Further patterns of fibrosis that extended transmurally was dependent on the transplant observed. This again is most likely a compensatory response to lower amounts of perfusion killing cardiomyocytes and activating fibroblasts to initiate collagen deposition. The intent of this mechanism is for the fibrous scar tissue to aid in structural integrity and prevent further damage.<sup>171</sup> Whole-heart cross-sections were analysed with ImageJ to provide a longitudinal assessment of fibrosis progression in the transplanted hearts. The quantified graph clearly depicts fibrosis increased in a stepwise manor with each timepoint, especially at day 28. Notably, day 28 transplants exhibited a 4.039 average fold-change from day 14 transplants, whereas day 7 transplants had a 1.730 average fold-change from day 3 transplants. These data demonstrate a fairly linear relationship between post-operative time and fibrosis accumulation within the transplanted hearts in antigen-matched animals.



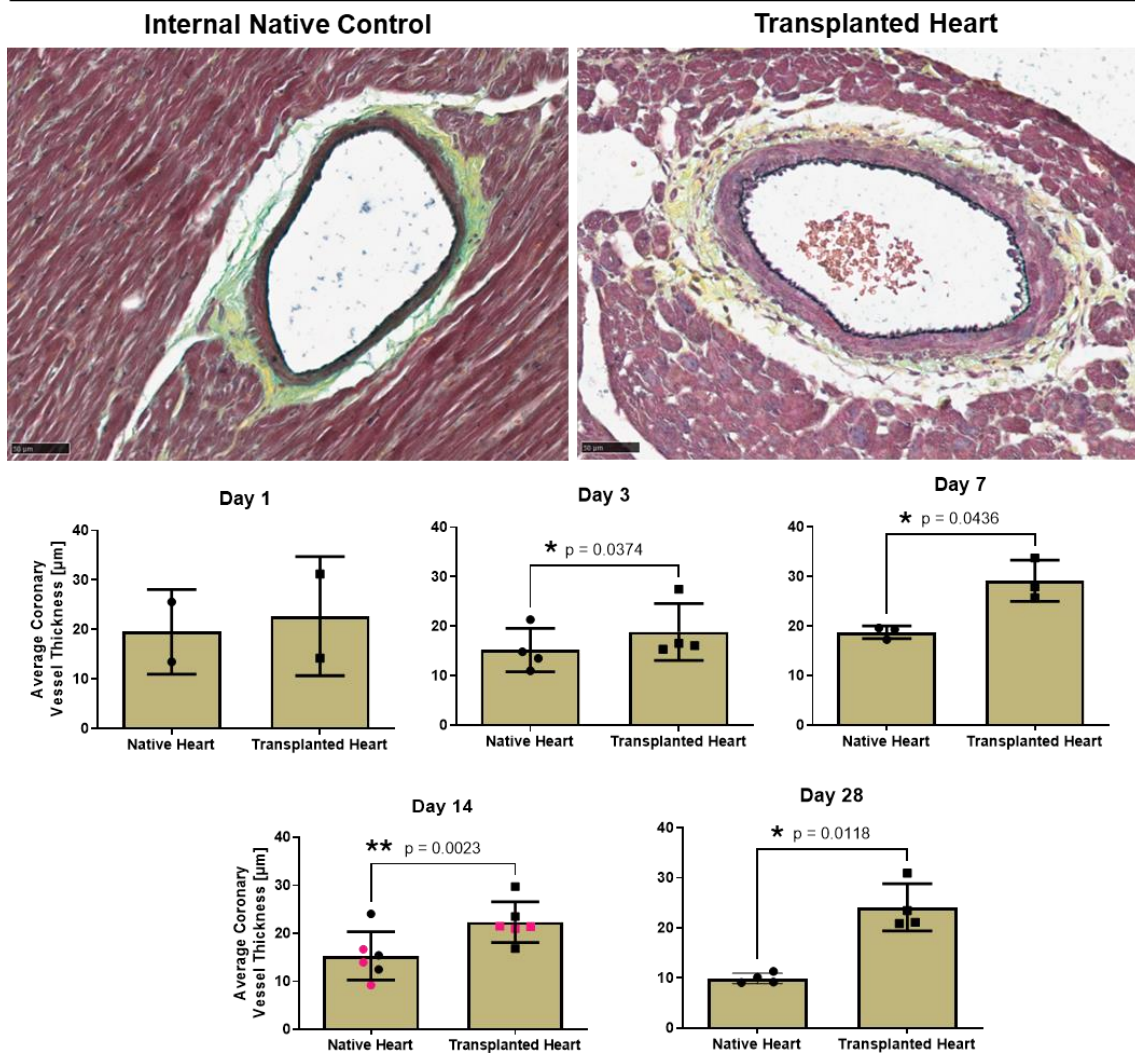
**Figure 15. Masson's Trichrome staining demonstrated increased fibrosis accumulation in the transplanted hearts over time.** Trichrome's aniline blue solution identified collagen on whole-heart cross sections that were quantified by color deconvolution on ImageJ. Black data points represent antigen-matched animals and pink data points represent antigen-mismatched animals. Enlarged image magnification = 20x, whole-heart image scale bar = 2.5 mm, magnified image scale bar = 100  $\mu$ m. The statistical

analysis performed was a one-way ANOVA with Tukey's post-hoc testing. Error bars indicate SD. \*\* $p < 0.01$  and \* $p < 0.05$ .

#### *4.4.4 Transplanted hearts exhibited coronary thickening indicative of transplant vasculopathy*

The main identifier for CAV in humans is the intimal thickening of the coronary arteries. While coronary arteries are not able to be collected for histologic analysis in the clinic, animal modeling allows us to obtain whole-heart cross-sections and quantify average coronary vessel thickness (Figure 16). Our objective was to distinguish if the transplanted hearts were developing any vasculopathy compared to their internal native controls. Movat's Pentachrome staining was adept at isolating the coronary arteries through the elastin stain shown as a thin black line decorating the interior walls of the vessels. ImageJ was utilized to measure vessel thickness and luminal area for all positively identified coronary arteries. Starting at day 3, average coronary thickness progressively increased with time in transplanted hearts compared to their internal native controls (Day 3,  $p = 0.0374$ ; Day 7,  $p = 0.0436$ ; Day 14,  $p = 0.0023$ ; Day 28,  $p = 0.0118$ ). By day 28, coronary vessel thickness consistently doubled in every transplanted heart compared to native counterparts. These findings support that the transplanted hearts are capable of developing some degree of vasculopathy even in their altered hemodynamic state.

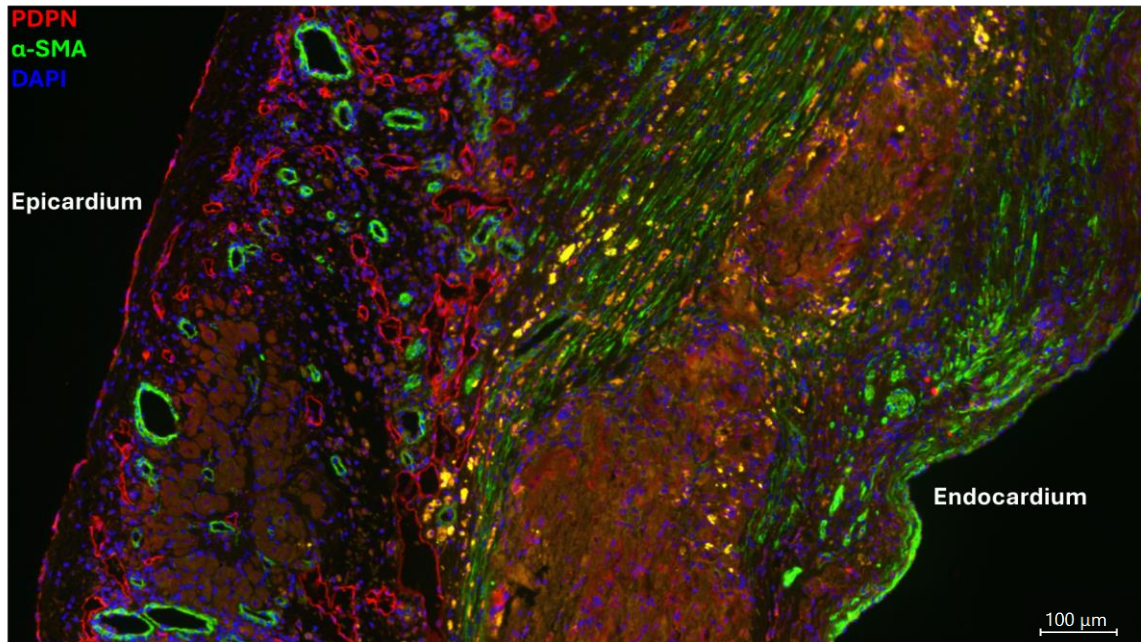
## Movat's Pentachrome



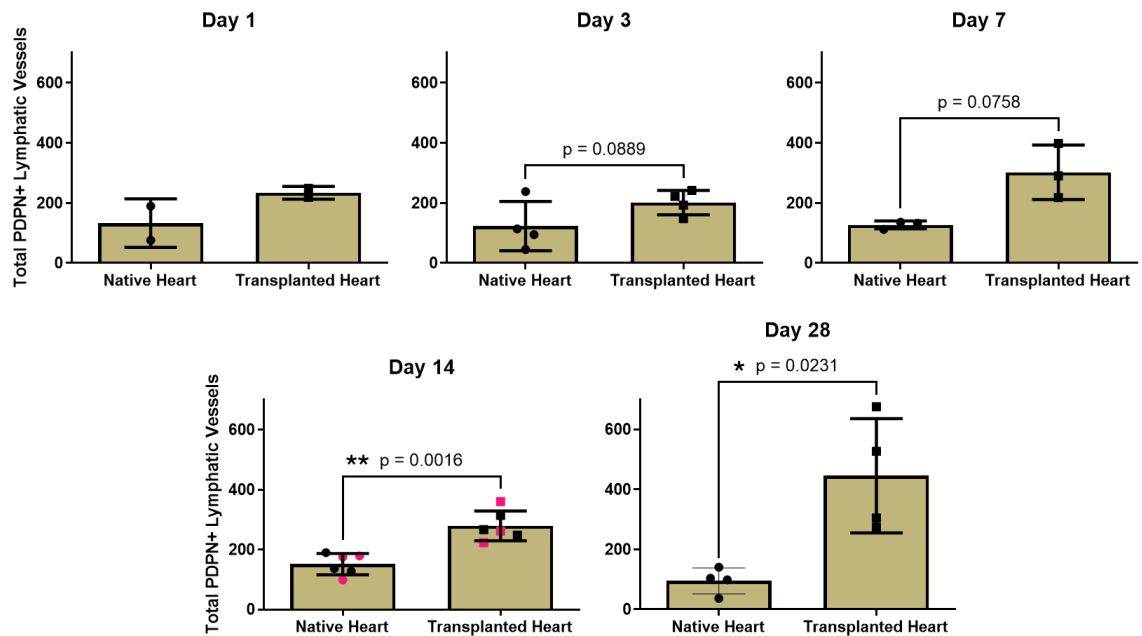
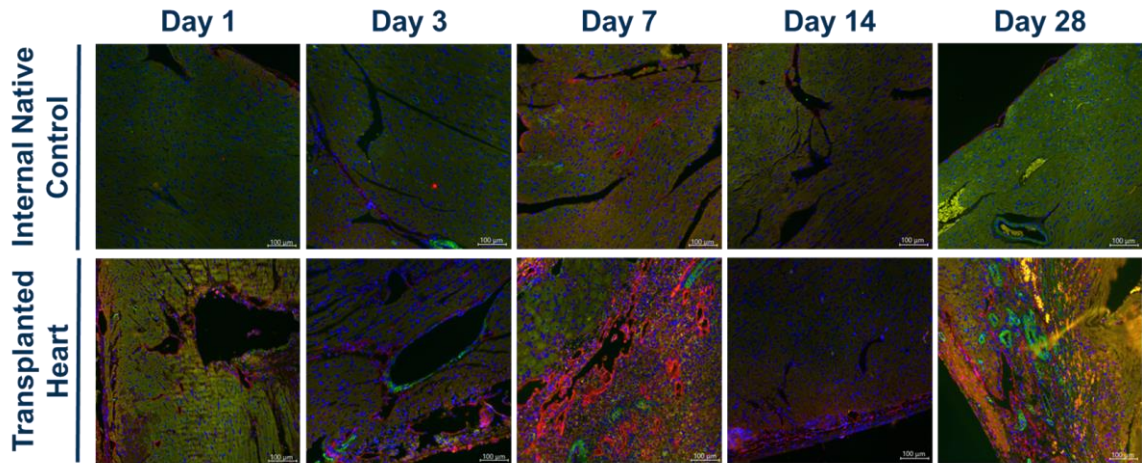
**Figure 16. Movat's Pentachrome staining exhibited increased average coronary vessel thickness in transplanted hearts compared to their internal native controls.** Pentachrome's elastin staining enabled identification of coronary arteries and quantification of vessel thickness. Black dots indicate antigen-matched transplants, while pink squares identify antigen mismatched transplants. Enlarged image magnification = 20x, whole-heart image scale bar = 2.5 mm, magnified image scale bar = 100  $\mu\text{m}$ . The statistical analysis performed was a two-way paired t-test. Error bars indicate SD. \*\* $p < 0.01$  and \* $p < 0.05$ .

#### 4.4.5 *Lymphatic density and luminal area increase after HAHT*

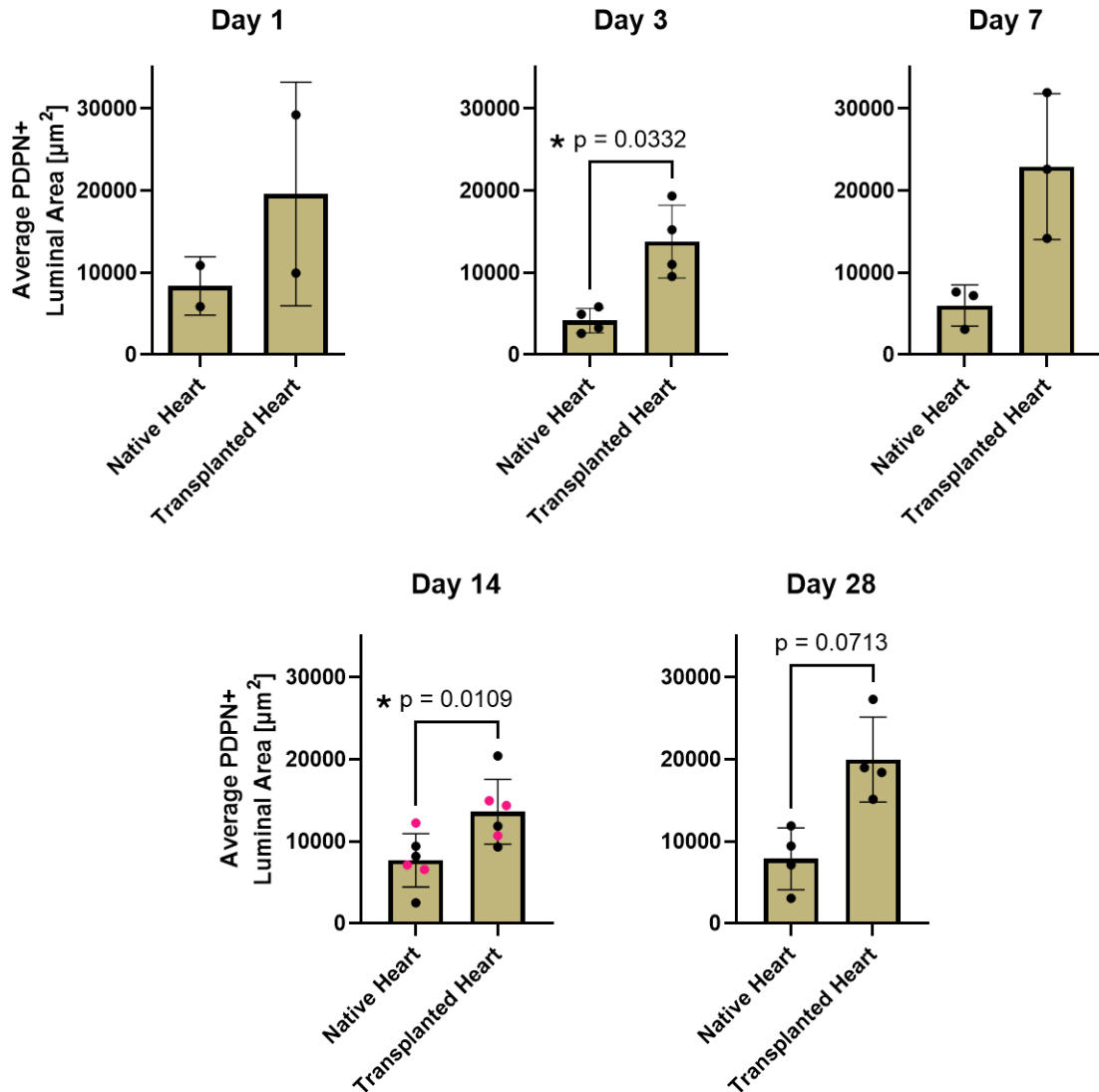
Immunofluorescent imaging was utilized to visualize both the blood and lymphatic vasculature with  $\alpha$ -SMA and PDPN respectively. These whole-heart cross-sectional images were imperative to characterize the longitudinal change in lymphatic quantity and structure over time. Qualitative observations of these tissue sections revealed the majority of the lymphatic vasculature was present in the epicardial space as is consistent with key physiological, anatomical, and functional properties of this microvascular system (Figure 17). The total number of lymphatics identified by PDPN+ signal was elevated in every transplanted heart but only significantly at day 14 ( $p = 0.0016$ ) and 28 ( $p = 0.0231$ ; Figure 18). Average lymphatic vessel number was highest in transplanted hearts at day 28 ( $446 \pm 165$ ) in comparison to day 14 ( $279 \pm 45$  total lymphatic vessels), day 7 ( $302 \pm 74$  total lymphatic vessels), day 3 ( $201 \pm 35$  total lymphatic vessels), and day 1 ( $234 \pm 15$  total lymphatic vessels) transplants. Furthermore, increased average lymphatic luminal area was most significant at day 14 ( $p = 0.0109$ ; Figure 19).



**Figure 17. Immunofluorescent imaging identified epicardial space to have a high prevalence of lymphatic vasculature compared to any other cardiac layer. PDPN = red;  $\alpha$ -SMA = green. Image was magnified 75% on Image J to show lymphatic distribution throughout the cardiac layers in a transplanted heart.**



**Figure 18. Lymphatic vasculature was significantly increased in transplanted hearts at later timepoints.** Immunofluorescent staining with lymphatic marker PDPN shown in red enabled the quantification of total lymphatic vessel number via ImageJ's Cell Counter plugin. Black data points represent antigen-matched animals and pink data points represent antigen-mismatched animals. The statistical analysis performed was a two-way paired t-test. Error bars indicate SD. \* $p < 0.05$ .

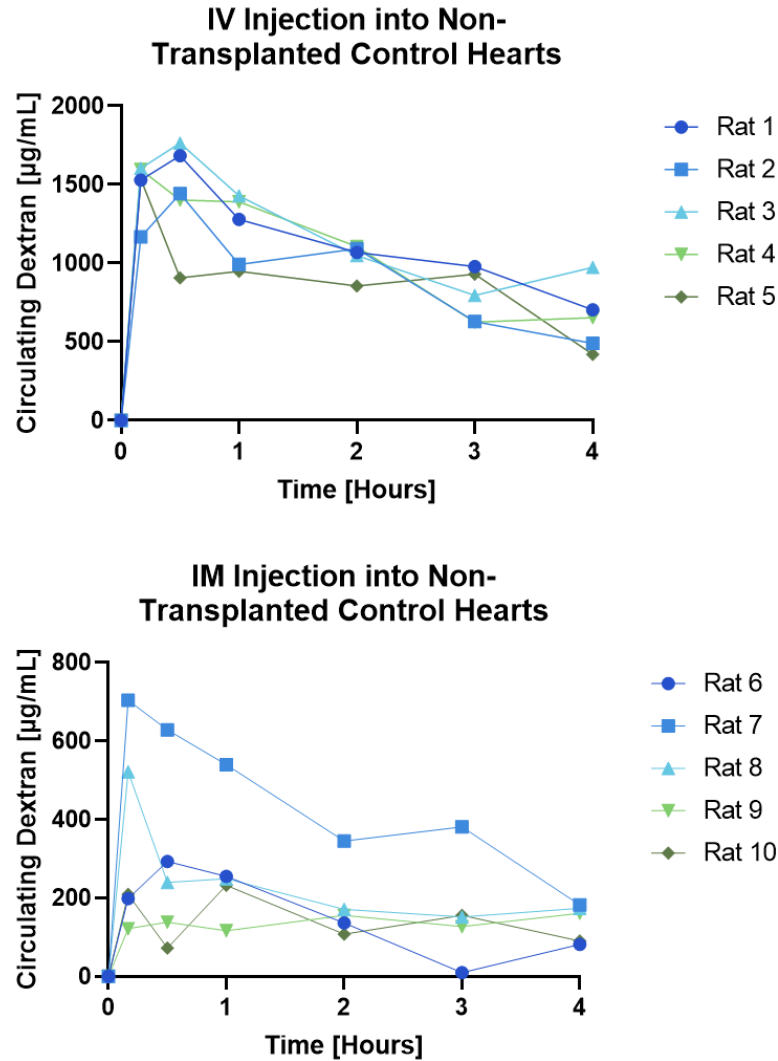


**Figure 19. Transplants exhibited increased PDPN+ luminal areas compared to internal native controls.** Immunofluorescent staining with lymphatic marker PDPN enabled the quantification of average lymphatic vessel luminal area via ImageJ. Black data points represent antigen-matched animals and pink data points represent antigen-mismatched animals. The statistical analysis performed was a two-way paired t-test. Error bars indicate SD. \* $p < 0.05$ .

#### 4.4.6 Lymphatic Drainage

Based on our immunofluorescent findings, we sought to determine whether the lymphatic restoration shown in the transplanted hearts was functional. While there are more

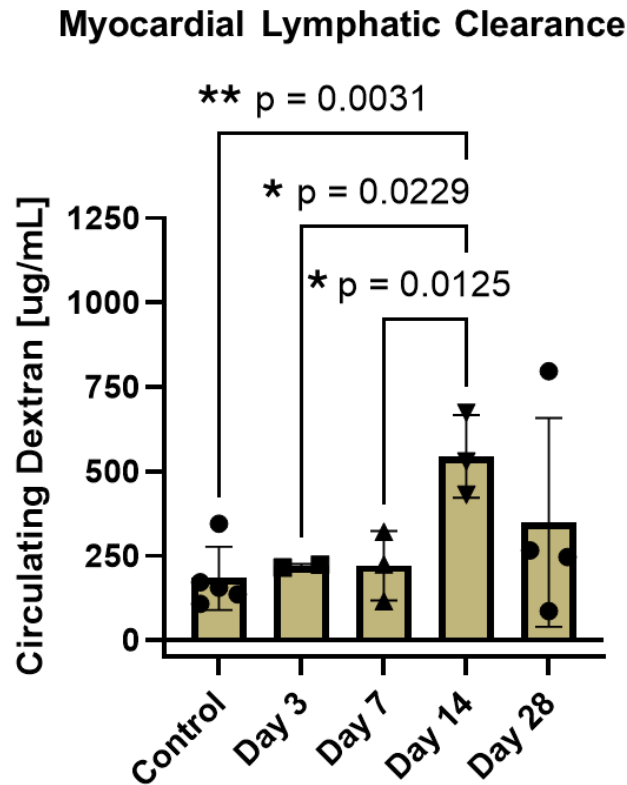
advanced methods that directly quantify lymphatic flow, our dextran assay provides rudimentary insight into the functional capacity of the transplanted hearts over time. To troubleshoot release profiles of the dextran-conjugated cyanine5.5, we utilized non-transplanted control animals for intravascular (IV) or intramyocardial (IM) injections (Figure 20). The IV injection was given through the left ventricle and served to define the release profiles of a bolus injection in the event that the intended intramyocardial injection was not conducted properly. IV injections revealed large spikes of circulating dextran at earlier timepoints, where averaged circulating dextran values at 10 and 30 minutes were  $1486 \pm 163$  and  $1440 \pm 300$   $\mu\text{g/mL}$  respectively. The IM injection gave a direct comparison of release profiles to transplant IM injections as a normalized non-transplanted control.



**Figure 20. Non-transplanted control animals served to contextualize tracer dynamics in vivo.**

These values were not only important to identify the degree of functional lymphatic drainage in the transplanted hearts but the timing of significant lymphatic involvement over the initial 4-hour time course. Based on the data, we determined that the 2-hour timepoint held the greatest influence of lymphatic involvement with the least amount of external confounding factors. Most timepoints had similar or higher levels of circulating dextran compared to the control for antigen-matched animals (Figure 21). However, the day 14

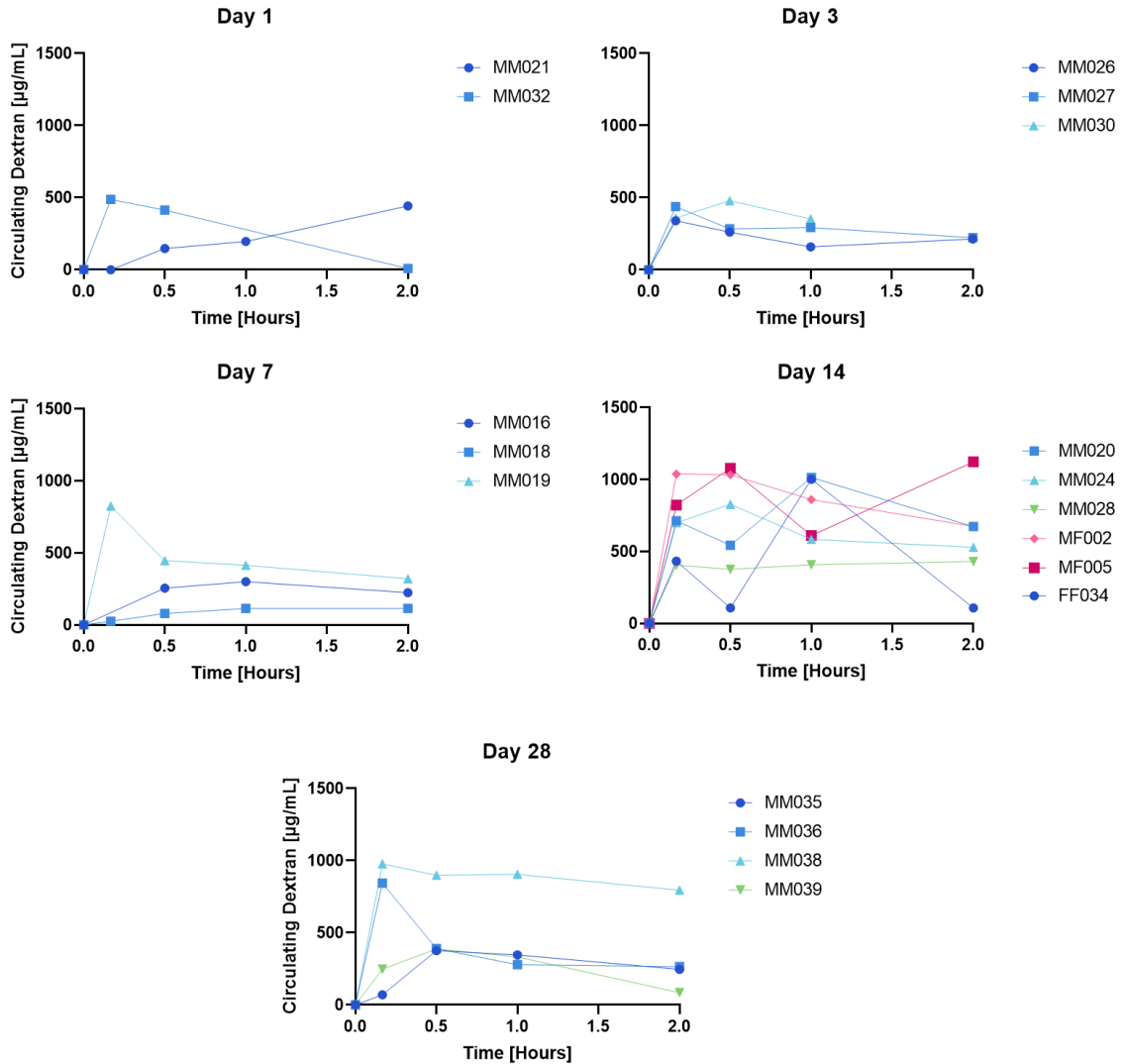
timepoint had a supranormal rate of drainage capabilities that was significant in comparison to all earlier timepoints (Control,  $p = 0.0031$ ; Day 3,  $p = 0.0229$ ; Day 7,  $p = 0.0125$ ). Day 14 is of particular interest to us as other solid-organ transplant models have shown functional lymphatic drainage via systemic reconnection occurs around 14 days.<sup>172</sup> Our data seems to support this timeline of functional lymphatic drainage occurring at day 14 within the transplanted hearts. It is reasonable to assume that the reduction of circulating dextran values seen at day 28 could be due to the increased fibrosis accumulation affecting lymphatic propulsion through a reduction in cardiac contractility.



**Figure 21.** Compiled antigen-matched tracer data at 2 hours demonstrated supranormal rate of lymphatic transport at day 14. The statistical analysis performed

was a one-way ANOVA with Tukey's post-hoc testing. Error bars indicate SD. \*\* $p < 0.01$  and \* $p < 0.05$ .

Longitudinal release profiles of every transplanted heart are shown in Figure 22. Although not significant, antigen-mismatched animal exhibited higher circulating dextran values at almost every collection timepoint compared to antigen-matched animals at day 14. Expansion of the antigen-mismatched cohort will need to be conducted before making any conclusive trends.

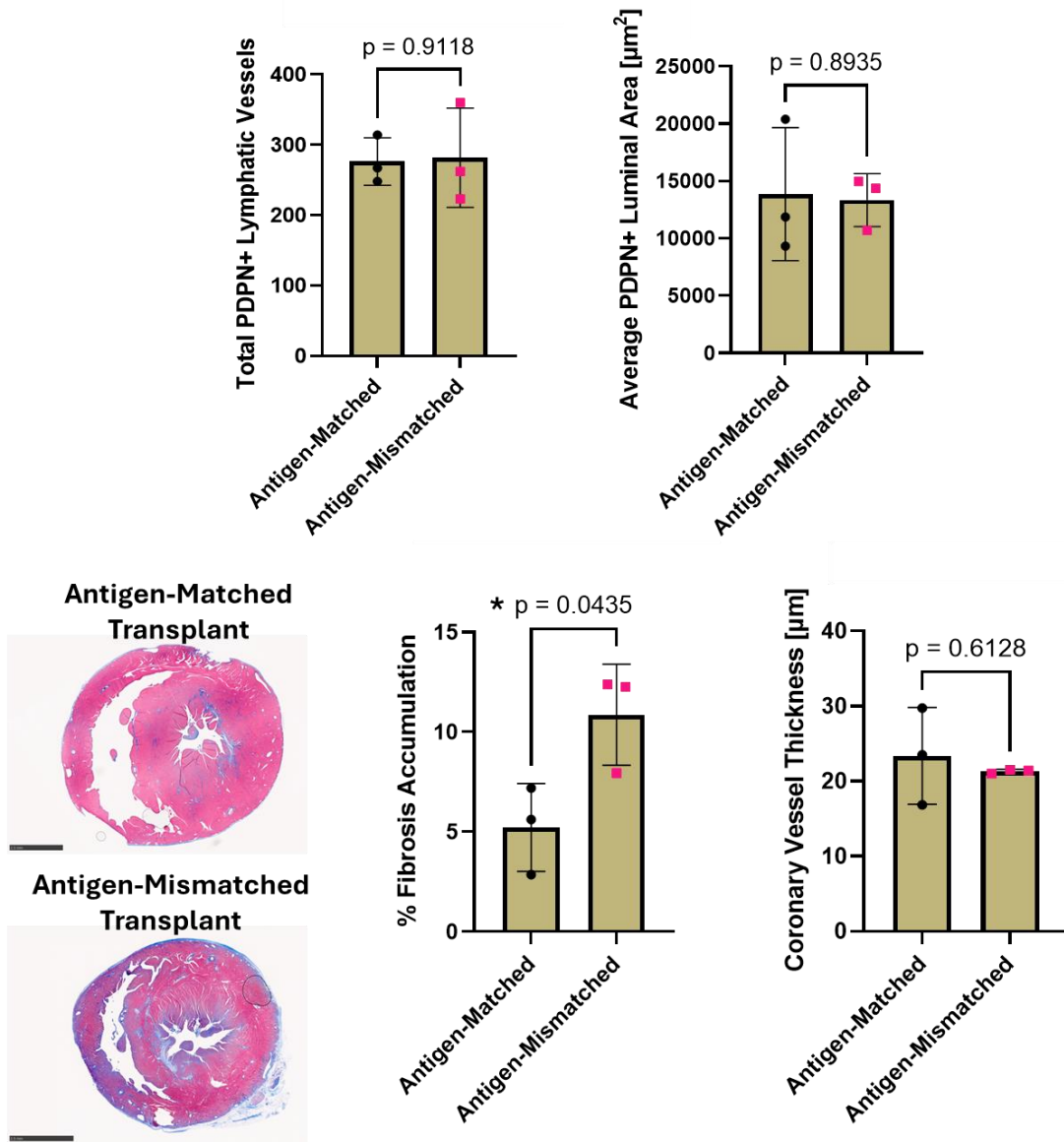


**Figure 22. Individual release profiles provided insight into lymphatic drainage capabilities of the transplanted hearts over a 2-hour time course at the time of sacrifice.**

#### 4.4.7 Antigen-mismatching yielded no differences in lymphatic vasculature

In preliminary attempts to induce rejection, we pursued a strain-mismatch model where Sprague-Dawley rat hearts would be implanted into LEW rats. This method proved to have an extremely accelerated rejection response that resulted in minimal survival. We suspect Sprague-Dawley being an outbred strain induced too much genetic variability that

inevitably mounted a massive immune response in the absence of immunosuppressive therapeutics. Pivoting our strategy, we then utilized a gender-mismatch model where male LEW rats were implanted into female LEW rats. This method introduces incompatibilities across minor histocompatibility complexes, where female recipients are known to have shorter survival and a higher risk of rejection due to the presence of Y-chromosome antigens.<sup>96</sup> The following data represents male-male antigen-matched and male-female antigen-mismatched animal cohorts at day 14 (Figure 23). There were no glaring differences in total PDPN+ lymphatic vessels, averaged PDPN+ luminal vessel area, or coronary thickness measurements between antigen-matched and antigen-mismatched transplanted hearts. However, there were differences in fibrosis accumulation when observing the whole-heart cross-sectional images. Antigen mismatched animals had significantly higher fibrosis percentages compared to their antigen-matched counterparts ( $p=0.0435$ ). Gender-based predispositions could potentially explain increased collagen accumulation in the transplanted hearts of female recipients but further investigations on how these predispositions affect metrics within this specific animal model need to be conducted.



**Figure 23. Gender-based antigen mismatching had increased fibrosis accumulation despite minimal differences in all other metrics at day 14.** The statistical analysis performed was a two-way unpaired t-test. Error bars indicate SD. \* $p < 0.05$ .

#### 4.5 Discussion

This study was able to successfully characterize longitudinal lymphatic changes and pathological remodeling in rat transplanted hearts. The goal of these experiments was to introduce lymphatics as a key player in transplant research and supply the framework to

study lymphatic influence on graft function. These data provide the foundational characterization to build future experimental studies manipulating the VEGF-C/VEGF-3 axis as a potential therapeutic target for transplant rejection.

Many pathological changes were observed over time in these transplanted hearts. The most obvious structural changes involved wall thinning and dilation at day 28. We found that the period between 14 and 28 days after HAHT was a period of increased remodeling driven by pathophysiologic factors not completely understood. While there is some myocardial volume loss from 0 to 14 days, it is accelerated by compounding factors of hemodynamic unloading over time. This reduction in myocardial volume seen at day 28 can impair the heart's ability to contract and pump blood efficiently. Our findings support a relatively linear increase in fibrosis with time post-HAHT which suggests a similar reduction in tissue compliance that is associated with the accelerated progression of heart failure clinically. Whole-heart imaging further supported this notion by revealing transplanted hearts at later timepoints had myocardial, interstitial, and perivascular fibrosis development. It is suspected that these factors of pathologic remodeling are to explain for increased size and weight of the transplanted heart from day 14 to day 28 in antigen-matched animals. Downstream consequences of volume overload and fibrosis accumulation offer promising metrics to rescue via lymphangiogenic intervention.

Predictably, our antigen-mismatched female recipients presented with significantly more fibrosis compared to our antigen-matched male recipients. This follows clinical trends describing how both gender-mismatching and female donor/recipients result in worse transplant outcomes.<sup>173,174</sup> These data can be attributed in part by gender-based differences in cardiovascular, immune, and physiologic responses. Estrogen especially

makes female rats more prone to rejection by enhancing the activity of helper T-cells and increasing the production of cytokines.<sup>175</sup> The resulting inflammation could be a principal activator of fibroblasts leading to excessive collagen accumulation. It is to be assumed that inducing rejection in antigen-mismatching through known models<sup>93</sup>, would also result in acute or chronic rejection and similarly accelerate these heart failure characteristics.

During this period of remodeling between 14 and 28 days, we also observed an increase in coronary vessel wall thickness in a process that histologically resembles CAV. The vessel thickening observed could potentially result in the ischemia responsible for the myocardial remodeling or additional factors including the maturation of the adaptive immune response. It is also intriguing that day 14 corresponds with a period of supraphysiologic lymphatic drainage, a time at which circulating immune cells could have increased rates of delivery to downstream lymph nodes. Both ischemic and immune factors contributing to cardiac remodeling suggest processes of rejection are active between 14 and 28 days. Although Pentachrome was sufficient in identifying significant increases of coronary thickness in the transplanted hearts, it is unable to look at the diffusivity of the lesion throughout the entirety of the vessel. The elastin stain is also not capable of identifying the intimal layer, only the elastic lamina of the coronaries. These restrictions leave us to report total vessel thickness instead of the more clinically relevant intimal thickness. Regardless, these data provided insight into the timeline of transplant vasculopathy in rodents and partially validate the translational impact of the model.

The dextran assay is a simple method to test the functionality of the restored lymphatic network. Our findings show a significant increase in lymphatic vasculature by histology post-HAHT but whether or not these lymphatics are functional will determine if

they will ameliorate or exacerbate graft dysfunction. Surprisingly, our dextran assay displayed relatively similar lymphatic transport at day 3 and 7 as our non-transplanted controls. This was unexpected as we were predicting systemic lymphatic reconstitution to occur around day 14. It is possible that lymphatic transport in completely healthy rat hearts is extremely low at baseline in comparison to diseased hearts and the similar levels seen at acute timepoints post-HAHT are not necessarily due to systemic lymphatic reconnection. Alternative drainage pathways could be compensating for the massive physiologic changes after a surgery this severe. A few different drainage pathways could explain how this tracer exited the myocardial tissue and entered circulation in an impaired lymphatic state. While the tracer is of a larger size to limit direct reabsorption into the blood vessels, it is possible that the tracer created abnormal pressure gradients and some minor microvascular damage that could account for the initial vasculature uptake and distribution to systemic circulation. This would be boosted in an inflammatory or stress state as vascular permeability might increase and allow larger amounts of the tracer to directly enter the bloodstream from the interstitial space. Moreover, there are other microvascular pathways that contribute to the return of substances to circulation. While not as prominent as lymphatic vasculature, these microvascular pathways have the capabilities to absorb interstitial fluid and/or tracer molecules. These possibilities would explain a significant elevation in circulating dextran during the time at which lymphatic reconstitution occurred. These data portray that timepoint of supranormal drainage to be day 14, where we believe the lymphatics have finally established systemic connections and compound the preexisting alternative drainage pathways. This timepoint of systemic lymphatic reconnection seems to serve as a turning point in pathological remodeling of the graft but it is still unclear what role the

lymphatics play in the graft's decline. These reestablished lymphatics could be enhancing immune cell trafficking to SLOs and initiating an inflammatory cascade that induces the resulting pathological remodeling seen at day 28. Conversely, the degree of lymphatic reconstitution seen could still be inadequate to transport pro-inflammatory cells and interstitial fluid that has accumulated over 28 days, where the demise of the graft is unsalvageable in a state of bottle-necked lymphatic transport. Overall, this assay gives a basic understanding of lymphatic transport capabilities but is limited in its ability to actually quantify the rate of tracer transport through the lymphatics. Future experiments would benefit from higher resolution *in vivo* imaging of the tracer in real-time utilizing systems like NIR imaging, positron emission tomography (PET), or even magnetic resonance imaging (MRI). These systems would vastly diversify quantification metrics (i.e. flow rates, regional drainage patterns, 3D mapping, etc.) but would come with substantial increases in cost and restricted availability.

The elevated abundance of lymphatics shown in these hearts can have many physiological and pathological implications. Lymphatics with functional drainage play a key role in removing excess interstitial fluid and metabolic waste products from the myocardium to improve tissue-fluid homeostasis. Additionally, these lymphatics maintain myocardial health by clearing toxins, lipids, inflammatory mediators, and dead cells. These actions may reflect the heart's attempt at healing itself where the lymphatics are central to the reparation process. Our data supports a significant increase in lymphatic vessels potentially in response initially to an impaired lymphatic system after surgery but sustained long-term due to the pathological remodeling present. The highest average lymphatic vessel number at day 28 could be an adaptive response triggered by tissue and pressure

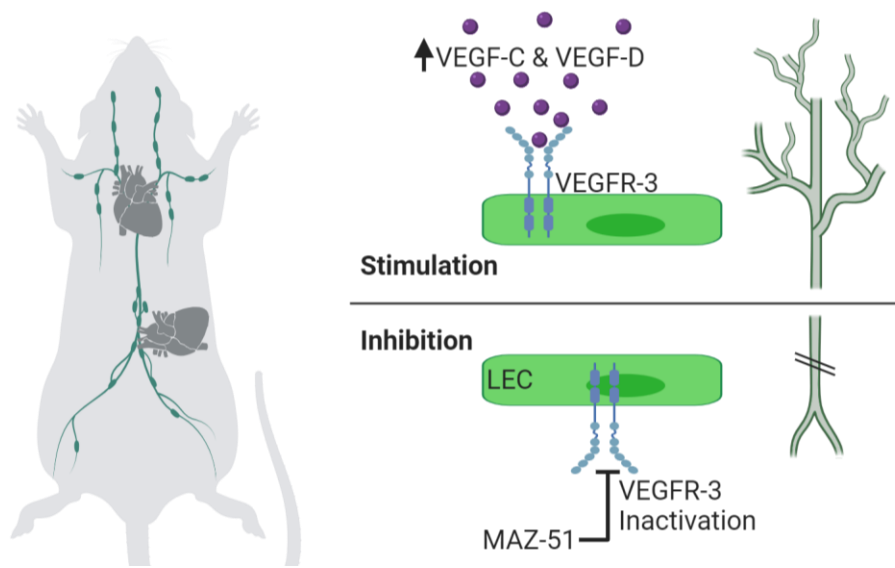
overload that caused the wall thinning and dilation seen in these transplanted hearts. However, excessive lymphatic growth in instances of chronic tissue stress could be indicative of a maladaptive tissue response rather than a beneficial adaptation. Furthering our understanding as to where this line crosses from beneficial to detrimental is imperative to contextualizing the role of lymphatics in stress, injury, and disease.

The elevated luminal areas of the lymphatic vessels are most likely an attempt to counteract fluid overload and increased tissue pressure. It stands to reason that the surgical insult alone would boost lymphangiogenesis but additional factors in the cardiac microenvironment may promote this regeneration long-term. Based on published literature, it is feasible to believe that up until day 14 there is minimal systemic lymphatic reconnections. The fluid overload present in these transplanted hearts would culminate over time leading to increased luminal areas until efficient routes of drainage were established. These large reservoir-like structures formed by the lymphatics are most likely exacerbated in earlier timepoints by improper drainage networks and in later timepoints by reduced cardiac contractility failing to efficiently propel fluid. These data clearly demonstrate that lymphatics in the transplanted heart have a sustained adaptive response. Further investigations altering this lymphangiogenic response will be necessary to understand the degree at which lymphatics affect transplant pathology, fluid dynamics, and immune function.

#### **4.6 Conclusions**

In summary, we established a rodent model of HTx that allowed us to characterize the adaptive abilities of lymphatic vasculature after surgery. Given the potential duality of

lymphatics in solid organ transplantation, our findings were imperative in determining a baseline response to surgical insult and pathologic changes over time. After HAHT, lymphatics experienced a sustained expansion in both quantity and size throughout the study duration. Since lymphatics are known to be key players in immune infiltration and tissue-fluid homeostasis, it is safe to assume these findings are a small part of a larger story detailing the heart's physiologic response to heal itself after injury. While our clinical study justified further investigations into lymphatic influence on transplant survival, this animal study has provided the characteristic foundation to now explore lymphatics as a potential prognostic marker and therapeutic target (Figure 24).



**Figure 24. Schematic of future studies manipulating the VEGF-C/VEGFR-3 axis to ameliorate rejection processes after HAHT.**

## CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

### 5.1 Conclusions

Lymphatics are an important component to any vascularized organ system. Their ability to modulate tissue-fluid homeostasis and immune cell trafficking has the ability to substantially influence many disease states including the leading cause of death, cardiovascular disease. While the vast amount of lymphatic research is centered around cancer, lymphatic diseases (e.g. lymphedema), and wound healing, we hope the research found within sparks interest in the role lymphatics play in a variety of cardiovascular disease states. The results of this dissertation address expansive gaps in cardiac lymphatic research in both the clinical and basic science setting. This research not only signifies lymphatics as an important metric in transplant survival but also furthers our understanding of lymphatic biology after HTx. Utilizing cardiac lymphatics as a therapeutic in transplantation research remains uncharted waters but offers a promising target with potentially extensive clinical impacts.

#### *5.1.1 Aim 1: Examine the longitudinal relationship between cardiac lymphatics, vascular remodeling, and survival in heart transplant patients*

We successfully implemented a retrospective clinical platform capable of assessing diverse metrics of transplant rejection and lymphatic presence in EMBs. We hypothesized that HTx recipients may have variable lymphatic adaptation after surgery leading to differences in graft function and survival. Our ability to quantify lymphatic area in EMBs allowed further stratification of the data into low and high lymphatic cohorts. These data

successfully correlated lower lymphatic areas to higher mortality long-term, warranting further investigations characterizing the role of lymphatics after HTx. The lymphatic quantification strategy presented also provides a promising potential biomarker to evaluate disease progression or even a screening tool for organs prior to procurement.

*5.1.2 Aim 2: Determine the impact of cardiac transplantation on lymphatic vasculature in a rodent model*

We established a HAHT model in rodents to explore lymphatic changes after HTx and its effect on graft function and survival. Our study design utilizing antigen-matched transplants enabled us to explore non-immunogenic effects of HAHT on cardiac lymphatics. Given the dual nature of lymphatics in solid organ transplantation, our findings were essential in determining a baseline response to surgical insult and pathologic changes over time. Transplanted hearts had both an increase in lymphatic density and lymphatic luminal area for extended periods after HAHT, where significant elevation in lymphatic drainage occurred at day 14 suggesting some degree of systemic lymphatic reconnection. We further identified this constrained period between 14 and 28 days as a key turning point in pathologic remodeling with vast therapeutic implications. Overall, these data provided critical insight into graft health in the context of lymphatic presence and cardiac remodeling. This research improves understanding in how lymphatic function is disrupted, maintained, and adapted after HAHT but more importantly provides a platform to control lymphangiogenic responses in an attempt to rescue function and survival long-term.

## 5.2 Future Directions

### 5.2.1 *Aim 1—Utilization of our clinical platform to explore lymphatic changes in the pediatric HTx*

Pediatric HTx presents itself with an entirely new set of challenges that differ significantly from adult HTx. The children affected are still growing and developing which requires a similar maturation of the cardiovascular system over time. It is not uncommon for pediatric patients to undergo multiple heart transplants in their lifetime. However, the longer lifespan of pediatric patients inevitably increases their risk of developing chronic rejection and CAV. Our clinical study has identified that lower amounts of lymphatics correlated to higher mortality in adult HTx patients. These findings at a minimum warrant lymphatics in EMBs to be considered as a prognostic biomarker for adverse transplant outcomes. Utilizing our established clinical platform, we stand to determine if similar correlations can be made in a pediatric setting. Moreover, this patient population is uniquely vulnerable to the long-term side effects of immunosuppression such as growth delays, infections, and cancer. Research into new therapeutic strategies targeted at either minimizing these side effects or inducing immune tolerance through alternative pathways would substantially improve long-term health concerns.

### 5.2.2 *Aim 1—Investigation of lymphatics and survival in the leading cause of acute graft dysfunction, primary graft dysfunction (PGD)*

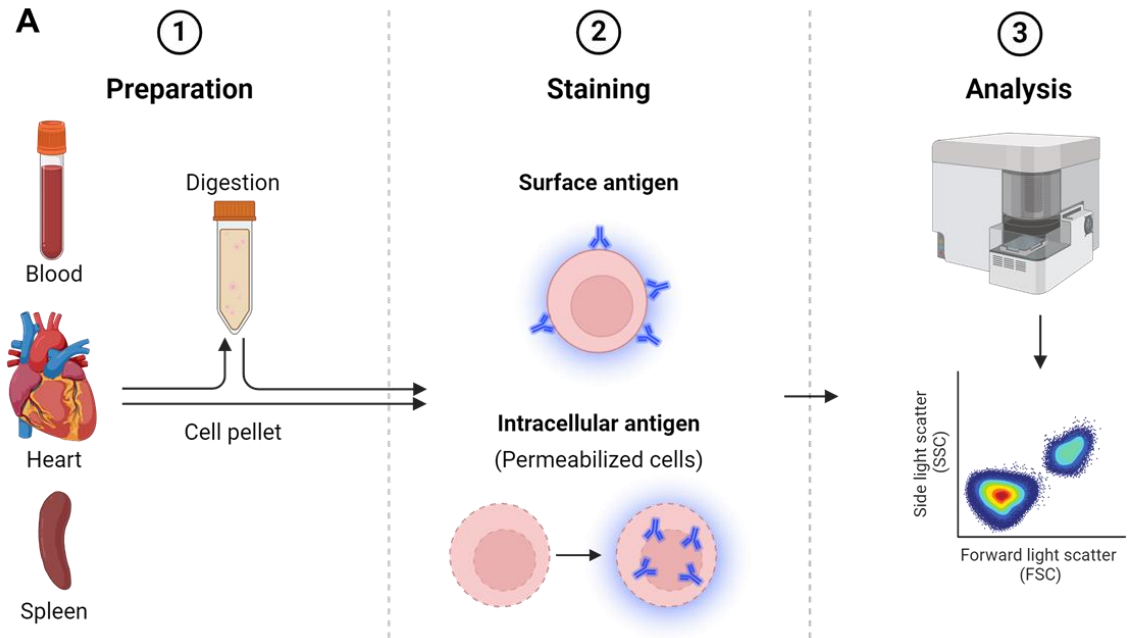
The clinical study described within focused on chronic timepoints post-HTx due to targeted adult CAV population. The main timepoints assessed being 1 and 5 years would have been well after the point of systemic lymphatic reconnection. However, more acute

timepoints may have impaired lymphatic drainage as a contributing factor to disease development and progression. PGD usually presents itself directly after surgery and remains the leading cause of early mortality in HTx patients.<sup>176</sup> This disease can cause single or biventricular dysfunction that results in failure to meet circulatory demands.<sup>176</sup> Where the inflammatory cascade following surgery is in part due to ischemia/reperfusion (I/R) injury, fluid accumulation, and tissue edema could be due to impaired lymphatic drainage. Many studies investigating pro-lymphangiogenic therapies for MI/R injury have concluded that lymphatic regeneration can reduce fluid retention, improve immune cell clearance, and decrease fibrosis in rodent models.<sup>18-22</sup> All of these factors could play a role in the progression of PGD. Studying PGD in the context of lymphatics would garner knowledge on how lymphatic dysfunction affects the early post-transplant period. Our findings could help identify risk factors of lymphatic congestion and serve as a sign to institute earlier interventions to prevent or treat PGD clinically. Future studies could also investigate I/R injury after HAHT and whether pro-lymphangiogenic therapies could mitigate I/R-induced inflammation and improve early graft dysfunction.

### *5.2.3 Aim 2—Further characterization of immunologic response after HAHT*

The inflammatory response is an extensive consideration in transplant research. One of the major strengths of this HAHT model is the clinically comparable immune response after HTx. Past studies were limited in the cardiac tissue available after sacrifice to have adequate cell numbers for flow cytometry. Future cohorts should utilize flow cytometry to link lymphatic clearance to immune cell infiltration by distinguishing prominent cell populations (i.e. macrophages, B-cells, T-cells, etc.) lingering in the transplanted and native hearts (Figure 25). The immune flow panel should consist of

live/dead, leukocytes (CD45), B-cells (CD45R), T-cells (CD4, CD8, and FoxP3), neutrophils (MPO), and macrophages (CD68). The lymphatic flow panel should consist of live/dead, endothelial cells (CD31), PDPN, and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1). The following panels could be stained utilizing i) recipient whole blood to determine circulating inflammatory cells levels, ii) recipient spleens to ascertain a systemic-based inflammatory response, and iii) recipient native and transplanted hearts for local representation of the inflammatory microenvironment and lymphatic presence. These panels will coincide with histological stainings described within to further support our findings. In addition, an enzyme-linked immunoassay (ELISA) assay could be utilized to obtain more sensitive measurements of a broad spectrum of identifiable proinflammatory markers (e.g. tumour necrosis factor- $\alpha$ , interleukin-6, chemokine ligand-21, etc.) and lymphatic markers (e.g. VEGF-C, VEGFR-3, PDPN, etc.). These kits prove to target physiologically relevant sensitivity levels and are widely available for our rodent model. As a whole, this immunologic characterization will further our knowledge on the fluctuations of immune cell populations after HTx and their impact on graft function and survival.



**B**

Panel	Marker	Antigen
Immune Panel	Leukocytes	CD45
	B-cells	CD45R
	T-cells	CD3
	Helper T-cells	CD4
	Cytotoxic T-cells	CD8
	Regulatory T-cells	FoxP3
	Neutrophils	MPO
	Macrophages	CD68
	Live/Dead	FVS620
Lymphatic Panel	Leukocytes	CD45
	Endothelial Cells	CD31
	Lymphatic Cells 1	PDPN
	Lymphatic Cells 2	LYVE-1
	Live/Dead	eFluor506

**Figure 25.** Experimental schematic of flow cytometry process (A) and panel design (B) utilized at pre-defined timepoints after HAHT.

#### 5.2.4 *Aim 2—Use of this rodent HAHT model to test lymphangiogenic therapies*

The use of this animal model to investigate lymphangiogenic therapies holds promise for improving graft survival, reducing rejection, and enhancing long-term transplant outcomes. Manipulating the VEGF-C/VEGFR-3 axis will better assess the role lymphatics play in graft tolerance. Stimulation of cardiac lymphatics in other cardiovascular disease states has proved beneficial to heart health and survival long-term.<sup>104,108,118,121,122</sup> Lymphatic regeneration is typically achieved by targeting lymphangiogenic growth factors (i.e. VEGF-C and VEGF-D) but modulating angiopoietins and the notch signalling pathway can also have similar effects. Inhibition of lymphatics in other cardiovascular models is a path far less travelled. The established benefits of augmenting lymphatic vasculature have minimized the study of lymphatic inhibition at large. However, the duality of lymphatics in HTx may prove lymphatic inhibition to be useful in future studies. Lymphatic inhibition can be achieved in many ways including but not limited to VEGF-C/VEGF-D blockades, VEGFR-3 inhibitors, blocking lymphangiogenic growth factors, notch signalling inhibition, and models of lymphatic vessel disruption. A large consideration for these studies will be the balance between inhibition and function. Over-inhibition has the potential to disrupt the balance of normal tissue repair and excessive fibrosis or inflammatory responses. Overall, any lymphangiogenic therapy considered should be highly specific and localized to avoid confounding factors of systemic involvement and comprising overall graft function. In that regard, biomaterials and gene delivery seem to be the future direction of cardiovascular delivery systems.

Future therapeutic studies should stimulate acute lymphatic regeneration to prevent the continual deterioration of cardiac allograft function caused by the chronic inflammatory

cascade. Lymphatic restoration will advocate for an immunomodulatory and reparative microenvironment, alleviating concerns of tissue-specific fluid build-up and immune cell trafficking after injury. However, repetitive protein injections into the myocardium often leads to rapid clearance that negates the effectiveness of the treatment<sup>177</sup> and large systemic doses of VEGF can cause adverse events (e.g. hemangiomas, diabetic retinopathy, atherosclerosis, cancer metastasis, etc.)<sup>178</sup>. To address these limitations, these studies should utilize a localized biomaterial-based approach through the incorporation of VEGF-C to promote pro-lymphangiogenic signalling and enhance inflammatory resolution immediately after cardiac transplantation. The characteristics of this hydrogel will enhance specificity by providing a localized delivery method that minimizes dose requirements, controls release, and protects VEGF-C from prior degradation. Utilizing these emerging concepts in the application of transplant rejection could dramatically effect specificity in treatment regimens while lowering off target side effects.

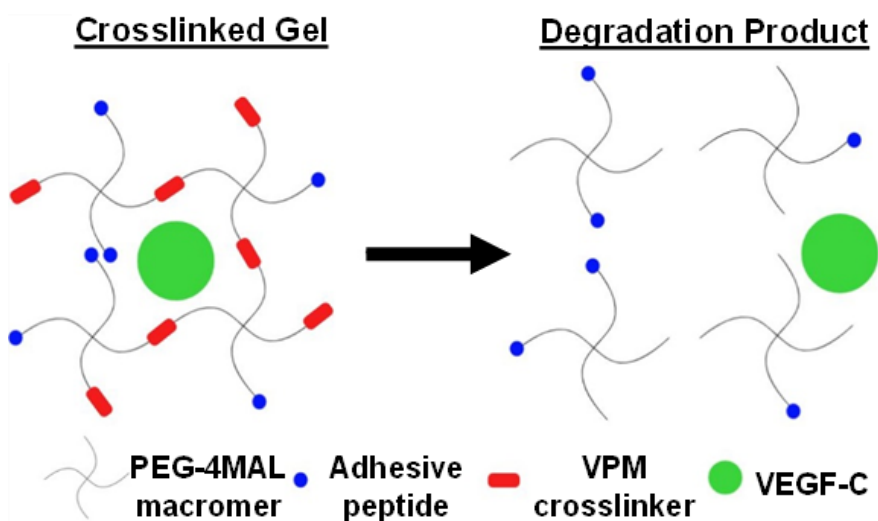
#### *5.2.5 Aim 2—Refinement of cardiac biomaterial as a therapeutic delivery mechanism*

Biomaterials are an easily modifiable strategy to deliver various therapeutics to enhance tissue repair and regeneration.<sup>179,180</sup> Several factors are considered in biomaterial design to optimize intended outcomes, including but not limited to i) biocompatibility to enhance functionality of biomaterial in its intended microenvironment while minimizing toxicity, immunogenicity, and foreign body responses associated with non-self materials, ii) composition and rigidity to tune cellular function and fate based on biological and mechanical properties, iii) porosity to influence cell-cell interactions and mass transport, iv) surface properties to promote cell adhesion, proliferation, and survival, and v) long-term application to develop a suitable biomaterial capable of sustaining the dynamic

complexities of its intended physiologic location. Synthetic hydrogels are ideal due to the following capabilities: large scale reproducibility, highly modifiable biomechanical properties, and customizable structure.<sup>177,181,182</sup> Biological biomaterials tend to have high lot-to-lot variability, potential toxicity, low biomechanical properties, and challenging structural complexity.<sup>180</sup> Poly(ethylene glycol) (PEG) is a synthetic material that has proven to be advantageous in numerous biomedical applications due to its inert physical properties and chemical/mechanical adaptability.<sup>179</sup> Their water-swollen 3D structure mimics environments similar to soft tissues and permits active diffusion of nutrients and cellular waste.<sup>179</sup> Due to the bioinert characteristics of PEG hydrogels limiting viability and integration into different tissue environments, four maleimide (MAL) groups functionalized onto the ends of a PEG macromer will promote tissue adherence through maleimide-thiol interactions.<sup>180</sup> The functionalization of maleimide groups occurs through Michael-type addition reactions that stoichiometrically incorporate adhesive properties at favorable polymerization speeds (<1min) and physiologic conditions (pH 6.5-7.5).<sup>180,182,183</sup>

These PEG-4MAL hydrogels have been successfully utilized as delivery vehicles intended to affect vascularization and regeneration through i) in vivo delivery of growth factors to promote vascularization, successful islet engraftment<sup>184</sup>, and bone regeneration<sup>185</sup> and ii) in vivo delivery of proteins and muscle stem cells to induce muscle regeneration<sup>180</sup>. Localized stimulation of a pro-lymphangiogenic factor (i.e. VEGF-C) provides a feasible option to combat limitations of relatively short VEGF family half-lives and undesirable off-target side effects of large bolus administrations.<sup>180,186</sup> The utilization of this biocompatible, functionalized PEG-4MAL hydrogel to deliver a continuous source of VEGF-C in vivo could locally enhance lymphangiogenesis, while concurrently reducing

myocardial edema and circulating immune cells/proinflammatory cytokines within the transplanted heart (Figure 26). Conversely, these biomaterials could be utilized to deliver MAZ-51, a VEGFR-3 inhibitor, to investigate the inflammatory milieu in the state of not only lymphatic inhibition but also lymphatic dysfunction directly after HAHT. Both stimulation and inhibition of lymphatic vasculature after HAHT would provide critical insight into the role of lymphatics in graft tolerance and pathological remodeling.



**Figure 26. Engineered design of a VEGF-C releasing hydrogel.**

5.2.6 *Aim 2—Investigation of complex interplay between immunosuppressive drugs and lymphangiogenic therapy*

A major factor influencing not only the health of the transplanted organ, but the patient as a whole is the need for long-term immune suppression. ISDs are the cornerstone of transplant therapeutics to prevent both acute and chronic rejection. Although long-term use of these drugs is synonymous with higher susceptibility in both infection and malignancy, their established beneficial nature in treatment regimens secure the utilization of these drugs for many years to come. Our current animal study excludes immune

suppression in an attempt to more accurately understand the natural inflammatory response after HAHT. While this is necessary in the early stages to make comparable assessments and validate therapeutic effects, future studies will need to incorporate ISDs into the treatment regimen to be more clinically applicable. The mechanistic diversity of these drugs will add to complexity of understanding the interaction between each ISD with a lymphangiogenic therapy when administered simultaneously.

## REFERENCES

- 1 B. Skorić, M. Č., J.L. Maček, Ž. Baričević, I. Škorak, H. Gašparović, B. Biočina, D. Miličić. Cardiac allograft vasculopathy: diagnosis, therapy, and prognosis. *Croatian Medical Journal* **28**, 412-414 (2014).
- 2 Chih, S., Chong, A. Y., Mielniczuk, L. M., Bhatt, D. L. & Beanlands, R. S. B. Allograft Vasculopathy: The Achilles' Heel of Heart Transplantation. *Journal of the American College of Cardiology* **68**, 80-91, doi:<https://doi.org/10.1016/j.jacc.2016.04.033> (2016).
- 3 Costello, J. P., Mohanakumar, T. & Nath, D. S. Mechanisms of chronic cardiac allograft rejection. *Tex. Heart Inst. J.* **40**, 395-399 (2013).
- 4 Schmauss, D. & Weis, M. Cardiac Allograft Vasculopathy. *Circulation* **117**, 2131-2141, doi:[doi:10.1161/CIRCULATIONAHA.107.711911](https://doi.org/10.1161/CIRCULATIONAHA.107.711911) (2008).
- 5 van den Hoogen, P., Huibers, M. M. H., Sluijter, J. P. G. & de Weger, R. A. Cardiac allograft vasculopathy: a donor or recipient induced pathology? *Journal of cardiovascular translational research* **8**, 106-116, doi:[10.1007/s12265-015-9612-x](https://doi.org/10.1007/s12265-015-9612-x) (2015).
- 6 Ramzy, D. *et al.* Cardiac allograft vasculopathy: a review. *Canadian journal of surgery. Journal canadien de chirurgie* **48**, 319-327 (2005).
- 7 Avery, R. K. Cardiac-Allograft Vasculopathy. *New England Journal of Medicine* **349**, 829-830, doi:[10.1056/NEJMp038124](https://doi.org/10.1056/NEJMp038124) (2003).
- 8 Elezaby, A., Dexheimer, R. & Sallam, K. Cardiovascular effects of immunosuppression agents. *Front Cardiovasc Med* **9**, 981838, doi:[10.3389/fcvm.2022.981838](https://doi.org/10.3389/fcvm.2022.981838) (2022).
- 9 Bartunek, J. *et al.* Cardiopoietic Stem Cell Therapy in Heart Failure. *Journal of the American College of Cardiology* **61**, 2329-2338, doi:[10.1016/j.jacc.2013.02.071](https://doi.org/10.1016/j.jacc.2013.02.071) (2013).
- 10 Che, Y.-J. *et al.* Lymph-Node-Targeted Drug Delivery for Effective Immunomodulation to Prolong the Long-Term Survival After Heart Transplantation. *Advanced Materials* **35**, 2207227, doi:<https://doi.org/10.1002/adma.202207227> (2023).
- 11 Dugbartey, G. J., Alornyo, K. K., Luke, P. P. W. & Sener, A. Application of carbon monoxide in kidney and heart transplantation: A novel pharmacological strategy

- for a broader use of suboptimal renal and cardiac grafts. *Pharmacol. Res.* **173**, 105883, doi:<https://doi.org/10.1016/j.phrs.2021.105883> (2021).
- 12 Bishawi, M. *et al.* A normothermic ex vivo organ perfusion delivery method for cardiac transplantation gene therapy. *Scientific Reports* **9**, 8029, doi:[10.1038/s41598-019-43737-y](https://doi.org/10.1038/s41598-019-43737-y) (2019).
  - 13 Bhagra, S. K., Pettit, S. & Parameshwar, J. Cardiac transplantation: indications, eligibility and current outcomes. *Heart* **105**, 252-260, doi:[10.1136/heartjnl-2018-313103](https://doi.org/10.1136/heartjnl-2018-313103) (2019).
  - 14 Valantine, H. Cardiac allograft vasculopathy after heart transplantation: risk factors and management. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **23**, S187-193, doi:[10.1016/j.healun.2004.03.009](https://doi.org/10.1016/j.healun.2004.03.009) (2004).
  - 15 Bråkenhielm, E., Chen, Y. & Cao, Y. Lymphatics in the broken heart. *The Journal of clinical investigation* **131**, doi:[10.1172/jci153448](https://doi.org/10.1172/jci153448) (2021).
  - 16 Aspelund, A., Robciuc, M. R., Karaman, S., Makinen, T. & Alitalo, K. Lymphatic System in Cardiovascular Medicine. *Circulation research* **118**, 515-530, doi:[10.1161/circresaha.115.306544](https://doi.org/10.1161/circresaha.115.306544) (2016).
  - 17 Dashkevich, A., Hagl, C., Beyersdorf, F., Nykanen, A. I. & Lemstrom, K. B. VEGF Pathways in the Lymphatics of Healthy and Diseased Heart. *Microcirculation* **23**, 5-14, doi:[10.1111/micc.12220](https://doi.org/10.1111/micc.12220) (2016).
  - 18 Klotz, L. *et al.* Cardiac lymphatics are heterogeneous in origin and respond to injury. *Nature* **522**, 62-67, doi:[10.1038/nature14483](https://doi.org/10.1038/nature14483) (2015).
  - 19 Henri, O. *et al.* Selective Stimulation of Cardiac Lymphangiogenesis Reduces Myocardial Edema and Fibrosis Leading to Improved Cardiac Function Following Myocardial Infarction. *Circulation* **133**, 1484-1497; discussion 1497, doi:[10.1161/circulationaha.115.020143](https://doi.org/10.1161/circulationaha.115.020143) (2016).
  - 20 Ishikawa, Y. *et al.* Lymphangiogenesis in myocardial remodelling after infarction. *Histopathology* **51**, 345-353, doi:[10.1111/j.1365-2559.2007.02785.x](https://doi.org/10.1111/j.1365-2559.2007.02785.x) (2007).
  - 21 Vieira, J. M. *et al.* The cardiac lymphatic system stimulates resolution of inflammation following myocardial infarction. *The Journal of clinical investigation* **128**, 3402-3412, doi:[10.1172/jci97192](https://doi.org/10.1172/jci97192) (2018).
  - 22 Norman, S. & Riley, P. R. Anatomy and development of the cardiac lymphatic vasculature: Its role in injury and disease. *Clin. Anat.* **29**, 305-315, doi:[10.1002/ca.22638](https://doi.org/10.1002/ca.22638) (2016).
  - 23 Chen, J. & Aronowitz, P. Congestive Heart Failure. *Med. Clin. North Am.* **106**, 447-458, doi:<https://doi.org/10.1016/j.mcna.2021.12.002> (2022).

- 24 Luo, Y., Liu, J., Zeng, J. & Pan, H. Global burden of cardiovascular diseases attributed to low physical activity: An analysis of 204 countries and territories between 1990 and 2019. *Am J Prev Cardiol* **17**, 100633, doi:10.1016/j.ajpc.2024.100633 (2024).
- 25 Bozkurt, B. *et al.* Heart Failure Epidemiology and Outcomes Statistics: A Report of the Heart Failure Society of America. *J. Card. Fail.* **29**, 1412-1451, doi:https://doi.org/10.1016/j.cardfail.2023.07.006 (2023).
- 26 Heidenreich, P. A. *et al.* 2022 AHA/ACC/HFSA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. *Circulation* **145**, e895-e1032, doi:10.1161/CIR.0000000000001063 (2022).
- 27 Simmonds, S. J., Cuijpers, I., Heymans, S. & Jones, E. A. V. Cellular and Molecular Differences between HFpEF and HFrEF: A Step Ahead in an Improved Pathological Understanding. *Cells* **9**, doi:10.3390/cells9010242 (2020).
- 28 Brink, J. G. & Hassoulas, J. The first human heart transplant and further advances in cardiac transplantation at Groote Schuur Hospital and the University of Cape Town - with reference to : the operation. A human cardiac transplant : an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town. *Cardiovasc. J. Afr.* **20**, 31-35 (2009).
- 29 Organ Procurement and Transplantation Network Metrics, 2024.
- 30 Nilsson, J. *et al.* The International Heart Transplant Survival Algorithm (IHTSA): A New Model to Improve Organ Sharing and Survival. *PLoS One* **10**, e0118644, doi:10.1371/journal.pone.0118644 (2015).
- 31 Madsen, J. C. Advances in the immunology of heart transplantation. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **36**, 1299-1305, doi:10.1016/j.healun.2017.10.003 (2017).
- 32 Ensor, C. R., Trofe-Clark, J., Gabardi, S., McDevitt-Potter, L. M. & Shullo, M. A. Generic Maintenance Immunosuppression in Solid Organ Transplant Recipients. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* **31**, 1111-1129, doi:https://doi.org/10.1592/phco.31.11.1111 (2011).
- 33 Singh, T. P. *et al.* Graft survival in primary thoracic organ transplant recipients: A special report from the International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation. *The Journal of Heart and Lung Transplantation* **42**, 1321-1333, doi:https://doi.org/10.1016/j.healun.2023.07.017 (2023).

- 34 Fallah, A. *et al.* Therapeutic targeting of angiogenesis molecular pathways in angiogenesis-dependent diseases. *Biomed. Pharmacother.* **110**, 775-785, doi:<https://doi.org/10.1016/j.biopha.2018.12.022> (2019).
- 35 Duncan, M. D. & Wilkes, D. S. Transplant-related immunosuppression: a review of immunosuppression and pulmonary infections. *Proc. Am. Thorac. Soc.* **2**, 449-455, doi:[10.1513/pats.200507-073JS](https://doi.org/10.1513/pats.200507-073JS) (2005).
- 36 Lee, H., Myoung, H. & Kim, S. M. Review of two immunosuppressants: tacrolimus and cyclosporine. *J Korean Assoc Oral Maxillofac Surg* **49**, 311-323, doi:[10.5125/jkaoms.2023.49.6.311](https://doi.org/10.5125/jkaoms.2023.49.6.311) (2023).
- 37 Mano, A. *et al.* Reversible myocardial hypertrophy induced by tacrolimus in a pediatric heart transplant recipient: case report. *Transplant. Proc.* **41**, 3831-3834, doi:[10.1016/j.transproceed.2009.05.040](https://doi.org/10.1016/j.transproceed.2009.05.040) (2009).
- 38 Kamiyoshi, Y. *et al.* Mycophenolate mofetil prevents the development of experimental autoimmune myocarditis. *J. Mol. Cell. Cardiol.* **39**, 467-477, doi:[10.1016/j.yjmcc.2005.04.004](https://doi.org/10.1016/j.yjmcc.2005.04.004) (2005).
- 39 Li, T. *et al.* Mycophenolate mofetil attenuates myocardial ischemia-reperfusion injury via regulation of the TLR4/NF- $\kappa$ B signaling pathway. *Pharmazie* **69**, 850-855 (2014).
- 40 Klawitter, J., Nashan, B. & Christians, U. Everolimus and sirolimus in transplantation-related but different. *Expert Opin. Drug Saf.* **14**, 1055-1070, doi:[10.1517/14740338.2015.1040388](https://doi.org/10.1517/14740338.2015.1040388) (2015).
- 41 Kushwaha, S. S. *et al.* Sirolimus affects cardiomyocytes to reduce left ventricular mass in heart transplant recipients. *Eur. Heart J.* **29**, 2742-2750, doi:[10.1093/eurheartj/ehn407](https://doi.org/10.1093/eurheartj/ehn407) (2008).
- 42 Imamura, T. *et al.* Everolimus Attenuates Myocardial Hypertrophy and Improves Diastolic Function in Heart Transplant Recipients. *Int. Heart J.* **57**, 204-210, doi:[10.1536/ihj.15-320](https://doi.org/10.1536/ihj.15-320) (2016).
- 43 Lindenfeld, J. *et al.* Drug Therapy in the Heart Transplant Recipient. *Circulation* **110**, 3858-3865, doi:[10.1161/01.CIR.0000150332.42276.69](https://doi.org/10.1161/01.CIR.0000150332.42276.69) (2004).
- 44 Park, M. H. *et al.* Oral steroid pulse without taper for the treatment of asymptomatic moderate cardiac allograft rejection. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **18**, 1224-1227, doi:[10.1016/s1053-2498\(99\)00098-4](https://doi.org/10.1016/s1053-2498(99)00098-4) (1999).
- 45 Stewart, S. *et al.* Revision of the 1990 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Heart Rejection. *The Journal of Heart and Lung Transplantation* **24**, 1710-1720, doi:[10.1016/j.healun.2005.03.019](https://doi.org/10.1016/j.healun.2005.03.019) (2005).

- 46 Gupta, S. *et al.* Utility of Routine Immunofluorescence Staining for C4d in Cardiac Transplant Recipients. *The Journal of Heart and Lung Transplantation* **28**, 776-780, doi:<https://doi.org/10.1016/j.healun.2009.05.007> (2009).
- 47 Glass, C., Butt, Y. M., Gokaslan, S. T. & Torrealba, J. R. CD68/CD31 immunohistochemistry double stain demonstrates increased accuracy in diagnosing pathologic antibody-mediated rejection in cardiac transplant patients. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **19**, 3149-3154, doi:[10.1111/ajt.15540](https://doi.org/10.1111/ajt.15540) (2019).
- 48 Shahandeh, N. *et al.* Invasive Coronary Imaging Assessment for Cardiac Allograft Vasculopathy: State-of-the-Art Review. *Journal of the Society for Cardiovascular Angiography & Interventions* **1**, 100344, doi:<https://doi.org/10.1016/j.jscai.2022.100344> (2022).
- 49 Wu, M. Y., Ali Khawaja, R. D. & Vargas, D. Heart Transplantation: Indications, Surgical Techniques, and Complications. *Radiol. Clin. North Am.* **61**, 847-859, doi:[10.1016/j.rcl.2023.04.011](https://doi.org/10.1016/j.rcl.2023.04.011) (2023).
- 50 Kobashigawa, J. *et al.* Report from a consensus conference on primary graft dysfunction after cardiac transplantation. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **33**, 327-340, doi:[10.1016/j.healun.2014.02.027](https://doi.org/10.1016/j.healun.2014.02.027) (2014).
- 51 Russo, M. J. *et al.* Factors associated with primary graft failure after heart transplantation. *Transplantation* **90**, 444-450, doi:[10.1097/TP.0b013e3181e6f1eb](https://doi.org/10.1097/TP.0b013e3181e6f1eb) (2010).
- 52 Segovia, J. *et al.* RADIAL: a novel primary graft failure risk score in heart transplantation. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **30**, 644-651, doi:[10.1016/j.healun.2011.01.721](https://doi.org/10.1016/j.healun.2011.01.721) (2011).
- 53 D'Ancona, G. *et al.* Primary graft failure after heart transplantation: the importance of donor pharmacological management. *Transplant. Proc.* **42**, 710-712, doi:[10.1016/j.transproceed.2010.03.027](https://doi.org/10.1016/j.transproceed.2010.03.027) (2010).
- 54 D'Alessandro, C. *et al.* Predictive risk factors for primary graft failure requiring temporary extra-corporeal membrane oxygenation support after cardiac transplantation in adults. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **40**, 962-969, doi:[10.1016/j.ejcts.2011.01.064](https://doi.org/10.1016/j.ejcts.2011.01.064) (2011).
- 55 Nicoara, A. *et al.* Primary graft dysfunction after heart transplantation: Incidence, trends, and associated risk factors. *Am. J. Transplant.* **18**, 1461-1470, doi:[10.1111/ajt.14588](https://doi.org/10.1111/ajt.14588) (2018).

- 56 Kumbala, D. & Zhang, R. Essential concept of transplant immunology for clinical practice. *World J Transplant* **3**, 113-118, doi:10.5500/wjt.v3.i4.113 (2013).
- 57 Kim, J. V. *et al.* Regulatory T Cell Biomarkers Identify Patients at Risk of Developing Acute Cellular Rejection in the First Year Following Heart Transplantation. *Transplantation* **107**, 1810-1819, doi:10.1097/tp.0000000000004607 (2023).
- 58 Angel A. Justiz Vaillant, S. M., Brian M. Fitzgerald. *Acute Transplant Rejection*. Jan. 2024 edn, (StatPearls Publishing 2024).
- 59 Chih, S. *et al.* Antibody-mediated rejection: an evolving entity in heart transplantation. *J Transplant* **2012**, 210210, doi:10.1155/2012/210210 (2012).
- 60 Rodriguez-Ramirez, S., Al Jurdi, A., Konvalinka, A. & Riella, L. V. Antibody-mediated rejection: prevention, monitoring and treatment dilemmas. *Curr Opin Organ Transplant* **27**, 405-414, doi:10.1097/mot.0000000000001011 (2022).
- 61 Wilhelm, M. J. Long-term outcome following heart transplantation: current perspective. *J. Thorac. Dis.* **7**, 549-551, doi:10.3978/j.issn.2072-1439.2015.01.46 (2015).
- 62 Lateef, N. *et al.* Malignancies After Heart Transplant. *Exp. Clin. Transplant.* **14**, 12-16, doi:10.6002/ect.2015.0214 (2016).
- 63 Rinaldi, M. *et al.* Neoplastic disease after heart transplantation: single center experience. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **19**, 696-701, doi:10.1016/s1010-7940(01)00674-1 (2001).
- 64 Roithmaier, S. *et al.* Incidence of malignancies in heart and/or lung transplant recipients: a single-institution experience. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **26**, 845-849, doi:10.1016/j.healun.2007.05.019 (2007).
- 65 Goldstein, D. J. *et al.* De novo solid malignancies after cardiac transplantation. *Ann. Thorac. Surg.* **60**, 1783-1789, doi:10.1016/0003-4975(95)00782-2 (1995).
- 66 O'Neill, J. O., Edwards, L. B. & Taylor, D. O. Mycophenolate Mofetil and Risk of Developing Malignancy After Orthotopic Heart Transplantation: Analysis of the Transplant Registry of the International Society for Heart and Lung Transplantation. *The Journal of Heart and Lung Transplantation* **25**, 1186-1191, doi:https://doi.org/10.1016/j.healun.2006.06.010 (2006).
- 67 Khush, K. K. *et al.* The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-sixth adult heart transplantation report — 2019; focus theme: Donor and recipient size match. *The*

- Journal of Heart and Lung Transplantation* **38**, 1056-1066, doi:<https://doi.org/10.1016/j.healun.2019.08.004> (2019).
- 68 Lund, L. H. *et al.* The Registry of the International Society for Heart and Lung Transplantation: Thirty-second Official Adult Heart Transplantation Report--2015; Focus Theme: Early Graft Failure. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **34**, 1244-1254, doi:10.1016/j.healun.2015.08.003 (2015).
- 69 Mrin Shetty, Y. S. C. (StatPearls Publishing, Treasure Island, FL, 2023).
- 70 Lu, W. H. *et al.* Diverse morphologic manifestations of cardiac allograft vasculopathy: a pathologic study of 64 allograft hearts. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **30**, 1044-1050, doi:10.1016/j.healun.2011.04.008 (2011).
- 71 Mehra, M. R. *et al.* International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy. *The Journal of Heart and Lung Transplantation* **29**, 717-727, doi:10.1016/j.healun.2010.05.017 (2010).
- 72 Gao, S. Z. *et al.* Acute myocardial infarction in cardiac transplant recipients. *Am. J. Cardiol.* **64**, 1093-1097, doi:10.1016/0002-9149(89)90858-8 (1989).
- 73 Zanchin, C. *et al.* Progression of cardiac allograft vasculopathy assessed by serial three-vessel quantitative coronary angiography. *PLoS One* **13**, e0202950, doi:10.1371/journal.pone.0202950 (2018).
- 74 Eisen, H. J. *et al.* Everolimus for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. *N. Engl. J. Med.* **349**, 847-858, doi:10.1056/NEJMoa022171 (2003).
- 75 Keogh, A. *et al.* Sirolimus in de novo heart transplant recipients reduces acute rejection and prevents coronary artery disease at 2 years: a randomized clinical trial. *Circulation* **110**, 2694-2700, doi:10.1161/01.Cir.0000136812.90177.94 (2004).
- 76 Eisen, H. J. *et al.* Everolimus versus mycophenolate mofetil in heart transplantation: a randomized, multicenter trial. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **13**, 1203-1216, doi:10.1111/ajt.12181 (2013).
- 77 Wenke, K. *et al.* Simvastatin reduces graft vessel disease and mortality after heart transplantation: a four-year randomized trial. *Circulation* **96**, 1398-1402, doi:10.1161/01.cir.96.5.1398 (1997).

- 78 Kobashigawa, J. A. *et al.* Effect of Pravastatin on Outcomes after Cardiac Transplantation. *New England Journal of Medicine* **333**, 621-627, doi:doi:10.1056/NEJM199509073331003 (1995).
- 79 Dandel, M. & Hetzer, R. Impact of immunosuppressive drugs on the development of cardiac allograft vasculopathy. *Curr. Vasc. Pharmacol.* **8**, 706-719, doi:10.2174/157016110792006923 (2010).
- 80 Choi, J. Y. *et al.* Regulatory CD8 T cells that recognize Qa-1 expressed by CD4 T-helper cells inhibit rejection of heart allografts. *Proceedings of the National Academy of Sciences of the United States of America*, doi:10.1073/pnas.1918950117 (2020).
- 81 Picarda, E. *et al.* Cross-Reactive Donor-Specific CD8(+) Tregs Efficiently Prevent Transplant Rejection. *Cell Rep.* **29**, 4245-4255.e4246, doi:10.1016/j.celrep.2019.11.106 (2019).
- 82 Śliwka, J. E., Tyrpień, M., Wilczek, P. M., Zembala, M. & Przybyłowski, P. A modified heterotopic heart transplantation in the rat - as an important model in experimental regeneration and replacement of the failing organ. *Kardiochir Torakochirurgia Pol* **17**, 149-154, doi:10.5114/kitp.2020.99079 (2020).
- 83 Benke, K. *et al.* Heterotopic Abdominal Rat Heart Transplantation as a Model to Investigate Volume Dependency of Myocardial Remodeling. *Transplantation* **101**, 498-505, doi:10.1097/tp.0000000000001585 (2017).
- 84 Szabó, G. *et al.* Catalytic peroxynitrite decomposition improves reperfusion injury after heart transplantation. *The Journal of thoracic and cardiovascular surgery* **143**, 1443-1449, doi:10.1016/j.jtcvs.2012.02.008 (2012).
- 85 Botha, P., MacGowan, G. A. & Dark, J. H. Sildenafil citrate augments myocardial protection in heart transplantation. *Transplantation* **89**, 169-177, doi:10.1097/TP.0b013e3181c42b22 (2010).
- 86 Wenzel, N., Blasczyk, R. & Figueiredo, C. Animal Models in Allogenic Solid Organ Transplantation. *Transplantology* **2**, 412-424 (2021).
- 87 Doenst, T., Schlensak, C., Kobba, J. L. & Beyersdorf, F. A technique of heterotopic, infrarenal heart transplantation with double anastomosis in mice. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **20**, 762-765 (2001).
- 88 Welsh, D. C. *et al.* Preserved contractile function despite atrophic remodeling in unloaded rat hearts. *Am. J. Physiol. Heart Circ. Physiol.* **281**, H1131-1136, doi:10.1152/ajpheart.2001.281.3.H1131 (2001).

- 89 Ito, K. *et al.* Contractile reserve and calcium regulation are depressed in myocytes from chronically unloaded hearts. *Circulation* **107**, 1176-1182, doi:10.1161/01.cir.0000051463.72137.96 (2003).
- 90 Kolár, F., MacNaughton, C., Papousek, F. & Korecky, B. Systolic mechanical performance of heterotopically transplanted hearts in rats treated with cyclosporin. *Cardiovasc. Res.* **27**, 1244-1247, doi:10.1093/cvr/27.7.1244 (1993).
- 91 Korecky, B., Ganguly, P. K., Elimban, V. & Dhalla, N. S. Muscle mechanics and Ca<sup>2+</sup> transport in atrophic heart transplants in rat. *Am. J. Physiol.* **251**, H941-948, doi:10.1152/ajpheart.1986.251.5.H941 (1986).
- 92 Madsen, J. C., Morris, P. J. & Wood, K. J. Immunogenetics of Heart Transplantation in Rodents. *Transplant. Rev.* **11**, 141-150, doi:https://doi.org/10.1016/S0955-470X(97)80014-6 (1997).
- 93 Bedi, D. S., Riella, L. V., Tullius, S. G. & Chandraker, A. Animal models of chronic allograft injury: contributions and limitations to understanding the mechanism of long-term graft dysfunction. *Transplantation* **90**, 935-944, doi:10.1097/TP.0b013e3181efcfbc (2010).
- 94 Adams, D. H. *et al.* Chronic rejection in experimental cardiac transplantation: studies in the Lewis-F344 model. *Immunol. Rev.* **134**, 5-19, doi:10.1111/j.1600-065x.1993.tb00637.x (1993).
- 95 Liu, X. *et al.* Macrophage Depletion Improves Chronic Rejection in Rats With Allograft Heart Transplantation. *Transplant. Proc.* **52**, 992-1000, doi:https://doi.org/10.1016/j.transproceed.2019.12.037 (2020).
- 96 Takami, H., Backer, C. L., Crawford, S. E., Zales, V. R. & Mavroudis, C. Influence of gender on allograft rejection in a rat heart transplant model. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **14**, 529-536 (1995).
- 97 Wong, B. W. Lymphatic vessels in solid organ transplantation and immunobiology. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, doi:10.1111/ajt.15806 (2020).
- 98 Hsu, M. C. & Itkin, M. Lymphatic Anatomy. *Techniques in vascular and interventional radiology* **19**, 247-254, doi:10.1053/j.tvir.2016.10.003 (2016).
- 99 Brakenhielm, E. & Alitalo, K. Cardiac lymphatics in health and disease. *Nature Reviews Cardiology* **16**, 56-68, doi:10.1038/s41569-018-0087-8 (2019).
- 100 Kim, H., Kataru, R. P. & Koh, G. Y. Inflammation-associated lymphangiogenesis: a double-edged sword? *The Journal of clinical investigation* **124**, 936-942, doi:10.1172/jci71607 (2014).

- 101 Kim, H., Kataru, R. P. & Koh, G. Y. Regulation and implications of inflammatory lymphangiogenesis. *Trends Immunol.* **33**, 350-356, doi:10.1016/j.it.2012.03.006 (2012).
- 102 Mouta, C. & Heroult, M. Inflammatory triggers of lymphangiogenesis. *Lymphat. Res. Biol.* **1**, 201-218, doi:10.1089/153968503768330247 (2003).
- 103 Deng, Y., Zhang, X. & Simons, M. Molecular controls of lymphatic VEGFR3 signaling. *Arterioscler. Thromb. Vasc. Biol.* **35**, 421-429, doi:10.1161/ATVBAHA.114.304881 (2015).
- 104 Vaahtomeri, K., Karaman, S., Makinen, T. & Alitalo, K. Lymphangiogenesis guidance by paracrine and pericellular factors. *Genes Dev.* **31**, 1615-1634, doi:10.1101/gad.303776.117 (2017).
- 105 Karkkainen, M. J. *et al.* Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* **5**, 74-80, doi:10.1038/ni1013 (2004).
- 106 Kataru, R. P. *et al.* T lymphocytes negatively regulate lymph node lymphatic vessel formation. *Immunity* **34**, 96-107, doi:10.1016/j.immuni.2010.12.016 (2011).
- 107 Oka, M. *et al.* Inhibition of endogenous TGF-beta signaling enhances lymphangiogenesis. *Blood* **111**, 4571-4579, doi:10.1182/blood-2007-10-120337 (2008).
- 108 Ou, J. *et al.* Endostatin suppresses colorectal tumor-induced lymphangiogenesis by inhibiting expression of fibronectin extra domain A and integrin  $\alpha 9$ . *J. Cell. Biochem.* **112**, 2106-2114, doi:10.1002/jcb.23130 (2011).
- 109 Cursiefen, C. *et al.* Thrombospondin 1 inhibits inflammatory lymphangiogenesis by CD36 ligation on monocytes. *J. Exp. Med.* **208**, 1083-1092, doi:10.1084/jem.20092277 (2011).
- 110 Randolph, G. J., Ivanov, S., Zinselmeyer, B. H. & Scallan, J. P. The Lymphatic System: Integral Roles in Immunity. *Annu. Rev. Immunol.* **35**, 31-52, doi:10.1146/annurev-immunol-041015-055354 (2017).
- 111 Norrmén, C., Tammela, T., Petrova, T. V. & Alitalo, K. Biological Basis of Therapeutic Lymphangiogenesis. *Circulation* **123**, 1335-1351, doi:doi:10.1161/CIRCULATIONAHA.107.704098 (2011).
- 112 Tammela, T. & Alitalo, K. Lymphangiogenesis: Molecular mechanisms and future promise. *Cell* **140**, 460-476, doi:10.1016/j.cell.2010.01.045 (2010).
- 113 Hirai, S. *et al.* Lymphangiogenesis in chronic inflammation in the testis. *Andrology* **1**, 147-154, doi:10.1111/j.2047-2927.2012.00015.x (2013).

- 114 Maruyama, K. *et al.* Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *The Journal of clinical investigation* **115**, 2363-2372, doi:10.1172/jci23874 (2005).
- 115 Kerjaschki, D. *et al.* Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. *Nat. Med.* **12**, 230-234, doi:10.1038/nm1340 (2006).
- 116 Lee, J. Y. *et al.* Podoplanin-expressing cells derived from bone marrow play a crucial role in postnatal lymphatic neovascularization. *Circulation* **122**, 1413-1425, doi:10.1161/circulationaha.110.941468 (2010).
- 117 Hall, K. L., Volk-Draper, L. D., Flister, M. J. & Ran, S. New model of macrophage acquisition of the lymphatic endothelial phenotype. *PLoS One* **7**, e31794, doi:10.1371/journal.pone.0031794 (2012).
- 118 Ratajska, A. *et al.* Comparative and Developmental Anatomy of Cardiac Lymphatics. *The Scientific World Journal* **2014**, 183170, doi:10.1155/2014/183170 (2014).
- 119 Shimada, T., Zhang, L., Abe, K., Yamabe, M. & Miyamoto, T. Developmental morphology of blood and lymphatic capillary networks in mammalian hearts, with special reference to three-dimensional architecture. *Ital. J. Anat. Embryol.* **106**, 203-211 (2001).
- 120 Juszyński, M., Ciszek, B., Stachurska, E., Jabłońska, A. & Ratajska, A. Development of lymphatic vessels in mouse embryonic and early postnatal hearts. *Developmental dynamics : an official publication of the American Association of Anatomists* **237**, 2973-2986, doi:10.1002/dvdy.21693 (2008).
- 121 Weirather, J. *et al.* Foxp3+ CD4+ T cells improve healing after myocardial infarction by modulating monocyte/macrophage differentiation. *Circulation research* **115**, 55-67, doi:10.1161/circresaha.115.303895 (2014).
- 122 Shore, L. R. The Lymphatic Drainage of the Human Heart. *J. Anat.* **63**, 291-313 (1929).
- 123 Johnson, R. A. & Blake, T. M. Lymphatics of the Heart. *Circulation* **33**, 137-142, doi:10.1161/01.CIR.33.1.137 (1966).
- 124 Dongaonkar, R. M., Stewart, R. H., Geissler, H. J. & Laine, G. A. Myocardial microvascular permeability, interstitial oedema, and compromised cardiac function. *Cardiovasc. Res.* **87**, 331-339, doi:10.1093/cvr/cvq145 (2010).
- 125 Klaourakis, K., Vieira, J. M. & Riley, P. R. The evolving cardiac lymphatic vasculature in development, repair and regeneration. *Nature Reviews Cardiology* **18**, 368-379, doi:10.1038/s41569-020-00489-x (2021).

- 126 Brakenhielm, E., González, A. & Díez, J. Role of Cardiac Lymphatics in Myocardial Edema and Fibrosis: JACC Review Topic of the Week. *Journal of the American College of Cardiology* **76**, 735-744, doi:https://doi.org/10.1016/j.jacc.2020.05.076 (2020).
- 127 Zheng, W., Aspelund, A. & Alitalo, K. Lymphangiogenic factors, mechanisms, and applications. *J. Clin. Invest.* **124**, 878-887, doi:10.1172/JCI71603 (2014).
- 128 Kajiya, K., Sawane, M., Huggenberger, R. & Detmar, M. Activation of the VEGFR-3 Pathway by VEGF-C Attenuates UVB-Induced Edema Formation and Skin Inflammation by Promoting Lymphangiogenesis. *J. Invest. Dermatol.* **129**, 1292-1298, doi:https://doi.org/10.1038/jid.2008.351 (2009).
- 129 Beaini, S. *et al.* VEGF-C attenuates renal damage in salt-sensitive hypertension. *J. Cell. Physiol.* **234**, 9616-9630, doi:https://doi.org/10.1002/jcp.27648 (2019).
- 130 Milasan, A., Smaani, A. & Martel, C. Early rescue of lymphatic function limits atherosclerosis progression in Ldlr<sup>-/-</sup> mice. *Atherosclerosis* **283**, 106-119, doi:https://doi.org/10.1016/j.atherosclerosis.2019.01.031 (2019).
- 131 Mehlhorn, U., Geissler, H. J., Laine, G. A. & Allen, S. J. Myocardial fluid balance. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **20**, 1220-1230, doi:10.1016/s1010-7940(01)01031-4 (2001).
- 132 Shimizu, Y. *et al.* Impact of Lymphangiogenesis on Cardiac Remodeling After Ischemia and Reperfusion Injury. *Journal of the American Heart Association* **7**, e009565, doi:10.1161/jaha.118.009565 (2018).
- 133 Vuorio, T. *et al.* Downregulation of VEGFR3 signaling alters cardiac lymphatic vessel organization and leads to a higher mortality after acute myocardial infarction. *Scientific Reports* **8**, 16709, doi:10.1038/s41598-018-34770-4 (2018).
- 134 Martinez-Corral, I. *et al.* In vivo imaging of lymphatic vessels in development, wound healing, inflammation, and tumor metastasis. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 6223-6228, doi:10.1073/pnas.1115542109 (2012).
- 135 Stacker, S. A. *et al.* Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nature Reviews Cancer* **14**, 159-172, doi:10.1038/nrc3677 (2014).
- 136 Banan, B. *et al.* Development of a novel murine model of lymphatic metastasis. *Clin. Exp. Metastasis*, doi:10.1007/s10585-020-10025-3 (2020).
- 137 Shimizu, Y. *et al.* Detection of lymphatic invasion in resected cases of primary pancreatic cancer based on immunohistochemistry of D2-40. *Annals of diagnostic pathology* **13**, 168-172, doi:10.1016/j.anndiagpath.2009.03.002 (2009).

- 138 Kumaravel, S. *et al.* CXCL11-CXCR3 Axis Mediates Tumor Lymphatic Cross Talk and Inflammation-Induced Tumor, Promoting Pathways in Head and Neck Cancers. *Am. J. Pathol.*, doi:10.1016/j.ajpath.2019.12.004 (2020).
- 139 Gelman, A. E. *et al.* Cutting edge: Acute lung allograft rejection is independent of secondary lymphoid organs. *J. Immunol.* **182**, 3969-3973, doi:10.4049/jimmunol.0803514 (2009).
- 140 Reed, H. O. *et al.* Lymphatic impairment leads to pulmonary tertiary lymphoid organ formation and alveolar damage. *The Journal of clinical investigation* **129**, 2514-2526, doi:10.1172/jci125044 (2019).
- 141 Li, W. *et al.* Bronchus-associated lymphoid tissue-resident Foxp3+ T lymphocytes prevent antibody-mediated lung rejection. *The Journal of clinical investigation* **129**, 556-568, doi:10.1172/jci122083 (2019).
- 142 Stucht, S. *et al.* Lymphatic neoangiogenesis in human renal allografts: results from sequential protocol biopsies. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **7**, 377-384, doi:10.1111/j.1600-6143.2006.01638.x (2007).
- 143 Pedersen, M. S. *et al.* Lymphangiogenesis in a mouse model of renal transplant rejection extends life span of the recipients. *Kidney Int.* **97**, 89-94, doi:10.1016/j.kint.2019.07.027 (2020).
- 144 Chih, S. *et al.* Fibrotic Plaque and Microvascular Dysfunction Predict Early Cardiac Allograft Vasculopathy Progression After Heart Transplantation: The Early Post Transplant Cardiac Allograft Vasculopathy Study. *Circ. Heart Fail.* **16**, e010173, doi:10.1161/circheartfailure.122.010173 (2023).
- 145 Seki, A. & Fishbein, M. C. Predicting the development of cardiac allograft vasculopathy. *Cardiovasc. Pathol.* **23**, 253-260, doi:10.1016/j.carpath.2014.05.001 (2014).
- 146 REICHERT, F. L. THE REGENERATION OF THE LYMPHATICS. *Arch. Surg.* **13**, 871-881, doi:10.1001/archsurg.1926.01130120095004 (1926).
- 147 Eraslan, S., Turner, M. D. & Hardy, J. D. LYMPHATIC REGENERATION FOLLOWING LUNG REIMPLANTATION IN DOGS. *Surgery* **56**, 970-973 (1964).
- 148 Baldwin, H. S. & Drakos, S. G. Lymphangiogenesis in Chronic Rejection and Coronary Allograft Vasculopathy: An Emerging Diagnostic and Therapeutic Target? *Circulation* **137**, 504-507, doi:10.1161/circulationaha.117.031716 (2018).
- 149 Edwards, L. A. *et al.* Chronic Rejection of Cardiac Allografts Is Associated With Increased Lymphatic Flow and Cellular Trafficking. *Circulation* **137**, 488-503, doi:10.1161/circulationaha.117.028533 (2018).

- 150 Dashkevich, A. *et al.* Ischemia-Reperfusion Injury Enhances Lymphatic Endothelial VEGFR3 and Rejection in Cardiac Allografts. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **16**, 1160-1172, doi:10.1111/ajt.13564 (2016).
- 151 Delgado, J. F. *et al.* Influence of cytomegalovirus infection in the development of cardiac allograft vasculopathy after heart transplantation. *The Journal of Heart and Lung Transplantation* **34**, 1112-1119, doi:https://doi.org/10.1016/j.healun.2015.03.015 (2015).
- 152 Ozeki, M. *et al.* Sirolimus treatment for intractable lymphatic anomalies: an open-label, single-arm, multicenter, prospective trial. *Front Med (Lausanne)* **11**, 1335469, doi:10.3389/fmed.2024.1335469 (2024).
- 153 Geissler, H. J. *et al.* First year changes of myocardial lymphatic endothelial markers in heart transplant recipients. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **29**, 767-771, doi:10.1016/j.ejcts.2005.12.024 (2006).
- 154 Balasubramanian, D. *et al.* Augmenting Renal Lymphatic Density Prevents Angiotensin II-Induced Hypertension in Male and Female Mice. *American journal of hypertension* **33**, 61-69, doi:10.1093/ajh/hpz139 (2020).
- 155 Tjang, Y. S., van der Heijden, G. J. M. G., Tenderich, G., Körfer, R. & Grobbee, D. E. Impact of Recipient's Age on Heart Transplantation Outcome. *The Annals of Thoracic Surgery* **85**, 2051-2055, doi:10.1016/j.athoracsur.2008.02.015 (2008).
- 156 Jaiswal, A., Kittleson, M., Pillai, A., Baran, D. & Baker, W. L. Usage of older donors is associated with higher mortality after heart transplantation: A UNOS observational study. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **43**, 806-815, doi:10.1016/j.healun.2024.01.005 (2024).
- 157 Waldron, R., Murray, C., Malpus, Z., Shearing, V. & Sanchez, M. The Experience of Heart Transplant as a Young Adult. *The Journal of Heart and Lung Transplantation* **32**, S196, doi:10.1016/j.healun.2013.01.481 (2013).
- 158 Wever-Pinzon, O. *et al.* Association of recipient age and causes of heart transplant mortality: Implications for personalization of post-transplant management—An analysis of the International Society for Heart and Lung Transplantation Registry. *The Journal of Heart and Lung Transplantation* **36**, 407-417, doi:https://doi.org/10.1016/j.healun.2016.08.008 (2017).
- 159 Murphy, E. & Kelly, D. P. Estrogen Signaling and Cardiovascular Disease. *Circ. Res.* **109**, 687-696, doi:10.1161/CIRCRESAHA.110.236687 (2011).

- 160 Stice, J. P., Lee, J. S., Pechenino, A. S. & Knowlton, A. A. Estrogen, aging and the cardiovascular system. *Future Cardiol.* **5**, 93-103, doi:10.2217/14796678.5.1.93 (2009).
- 161 Crandall, B. G. *et al.* Increased cardiac allograft rejection in female heart transplant recipients. *J. Heart Transplant.* **7**, 419-423 (1988).
- 162 DeFilippis, E. M., Nikolova, A., Holzhauser, L. & Khush, K. K. Understanding and Investigating Sex-Based Differences in Heart Transplantation: A Call to Action. *JACC: Heart Failure* **11**, 1181-1188, doi:https://doi.org/10.1016/j.jchf.2023.06.030 (2023).
- 163 Fontaine, C. *et al.* The Impact of Estrogen Receptor in Arterial and Lymphatic Vascular Diseases. *Int. J. Mol. Sci.* **21**, doi:10.3390/ijms21093244 (2020).
- 164 Boucquemont, J. *et al.* Gender Differences in Medication Adherence Among Adolescent and Young Adult Kidney Transplant Recipients. *Transplantation* **103**, 798-806, doi:10.1097/tp.0000000000002359 (2019).
- 165 Morris, A. A., Kransdorf, E. P., Coleman, B. L. & Colvin, M. Racial and ethnic disparities in outcomes after heart transplantation: A systematic review of contributing factors and future directions to close the outcomes gap. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **35**, 953-961, doi:10.1016/j.healun.2016.01.1231 (2016).
- 166 Westhofen, S. *et al.* The heterotopic heart transplantation in mice as a small animal model to study mechanical unloading - Establishment of the procedure, perioperative management and postoperative scoring. *PLoS One* **14**, e0214513, doi:10.1371/journal.pone.0214513 (2019).
- 167 Lim, S. H. *et al.* Adenosine injection prior to cardioplegia enhances preservation of senescent hearts in rat heterotopic heart transplantation. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **43**, 1202-1208, doi:10.1093/ejcts/ezs509 (2013).
- 168 Szabó, G. *et al.* Immunomodulatory effects of poly(ADP-ribose) polymerase inhibition contribute to improved cardiac function and survival during acute cardiac rejection. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **25**, 794-804, doi:10.1016/j.healun.2006.03.017 (2006).
- 169 Panther, F. *et al.* Inhibition of nuclear translocation of calcineurin suppresses T-cell activation and prevents acute rejection of donor hearts. *Transplantation* **91**, 597-604, doi:10.1097/TP.0b013e3182090f67 (2011).
- 170 Brieler, J., Breeden, M. A. & Tucker, J. Cardiomyopathy: An Overview. *Am. Fam. Physician* **96**, 640-646 (2017).

- 171 Travers, J. G., Kamal, F. A., Robbins, J., Yutzey, K. E. & Blaxall, B. C. Cardiac Fibrosis. *Circ. Res.* **118**, 1021-1040, doi:10.1161/CIRCRESAHA.115.306565 (2016).
- 172 Ruggiero, R. *et al.* Reestablishment of lymphatic drainage after canine lung transplantation. *The Journal of thoracic and cardiovascular surgery* **106**, 167-171 (1993).
- 173 Previato, M., Osto, E., Kerkhof, P. L. M., Parry, G. & Tona, F. Heart Transplantation Survival and Sex-Related Differences. *Adv. Exp. Med. Biol.* **1065**, 379-388, doi:10.1007/978-3-319-77932-4\_24 (2018).
- 174 Morgan, A. E. *et al.* The role of estrogen, immune function and aging in heart transplant outcomes. *The American Journal of Surgery* **218**, 737-743, doi:https://doi.org/10.1016/j.amjsurg.2019.07.007 (2019).
- 175 Savolainen, H., Frösen, J., Petrov, L., Aavik, E. & Häyry, P. Expression of estrogen receptor sub-types alpha and beta in acute and chronic cardiac allograft vasculopathy. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* **20**, 1252-1264, doi:10.1016/s1053-2498(01)00363-1 (2001).
- 176 Al-Adhami, A. *et al.* Primary Graft Dysfunction after Heart Transplantation - Unravelling the Enigma. *Curr. Probl. Cardiol.* **47**, 100941, doi:10.1016/j.cpcardiol.2021.100941 (2022).
- 177 García, A. J. PEG–Maleimide Hydrogels for Protein and Cell Delivery in Regenerative Medicine. *Ann. Biomed. Eng.* **42**, 312-322, doi:10.1007/s10439-013-0870-y (2014).
- 178 Salimath, A. S. *et al.* Dual Delivery of Hepatocyte and Vascular Endothelial Growth Factors via a Protease-Degradable Hydrogel Improves Cardiac Function in Rats. *PLoS One* **7**, e50980, doi:10.1371/journal.pone.0050980 (2012).
- 179 Zhu, J. Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering. *Biomaterials* **31**, 4639-4656, doi:10.1016/j.biomaterials.2010.02.044 (2010).
- 180 Han, W. M., Mohiuddin, M., Anderson, S. E., García, A. J. & Jang, Y. C. Co-delivery of Wnt7a and muscle stem cells using synthetic bioadhesive hydrogel enhances murine muscle regeneration and cell migration during engraftment. *Acta Biomater.* **94**, 243-252, doi:https://doi.org/10.1016/j.actbio.2019.06.025 (2019).
- 181 Han Woojin, M. *et al.* Synthetic matrix enhances transplanted satellite cell engraftment in dystrophic and aged skeletal muscle with comorbid trauma. *Science Advances* **4**, eaar4008, doi:10.1126/sciadv.aar4008.

- 182 Phelps, E. A. *et al.* Maleimide cross-linked bioactive PEG hydrogel exhibits improved reaction kinetics and cross-linking for cell encapsulation and in situ delivery. *Adv Mater* **24**, 64-62, doi:10.1002/adma.201103574 (2012).
- 183 Hiemstra, C., van der Aa, L. J., Zhong, Z., Dijkstra, P. J. & Feijen, J. Rapidly in Situ-Forming Degradable Hydrogels from Dextran Thiols through Michael Addition. *Biomacromolecules* **8**, 1548-1556, doi:10.1021/bm061191m (2007).
- 184 Phelps, E. A., Templeman, K. L., Thule, P. M. & Garcia, A. J. Engineered VEGF-releasing PEG-MAL hydrogel for pancreatic islet vascularization. *Drug Deliv Transl Res* **5**, 125-136, doi:10.1007/s13346-013-0142-2 (2015).
- 185 Garcia, J. R., Clark, A. Y. & Garcia, A. J. Integrin-specific hydrogels functionalized with VEGF for vascularization and bone regeneration of critical-size bone defects. *J. Biomed. Mater. Res. A* **104**, 1845, doi:10.1002/jbm.a.35777 (2016).
- 186 Campbell, K. T., Hadley, D. J., Kukis, D. L. & Silva, E. A. Alginate hydrogels allow for bioactive and sustained release of VEGF-C and VEGF-D for lymphangiogenic therapeutic applications. *PLoS One* **12**, e0181484, doi:10.1371/journal.pone.0181484 (2017).