

**FUNCTIONAL AND STRUCTURAL REMODELING IN HEART FAILURE:
EVALUATION OF RYANODINE RECEPTOR 2 AND JUNCTOPHILIN 2 AS A
POTENTIAL BIOMARKER OF CARDIAC RECOVERY IN PATIENTS WITH LEFT
VENTRICLE ASSIST DEVICES**

**Younjee Lee^{1,2} (Frank B. Sachse, Ph.D. ^{1,2})
Department of Biomedical Engineering**

Abstract—Left ventricular assist devices (LVADs) are mechanical pumps that are implanted in end-stage heart failure (HF) patients as a bridge to heart transplantation or destination therapy. In some cases, patients respond to LVAD implantation with cardiac recovery, and these patients no longer need a heart transplant. The aim of our study was to establish a biomarker for cardiac recovery after LVAD unloading based on the tissue microstructure. In particular, we studied the spatial distribution of ryanodine receptor 2 (RyR2) and junctophilin (JPH2) as well as the transverse tubular system (t-system). Assuming that proper localization of RyR2 and JPH2 to the t-system is crucial for cardiomyocyte function, we hypothesized that only patients without remodeling of microstructural features recover their cardiac function after LVAD unloading. We applied 3-dimensional microscopy and computational image analyses to compare RyR2 and JPH2 localization in donor tissues (n=8) as well as HF tissues with (n=10) and without recovery (n=5) after LVAD unloading. The t-system in HF patients without recovery exhibited sparse and sheet-like remodeling as previously reported (Seidel et al, Circ, 2017). Protein distributions were specifically altered in tissues from HF patients without recovery versus healthy donor tissues. In HF tissues without recovery, JPH2 and RyR2 were distributed at higher distances from sarcolemma. However, the cell microstructure in patients with cardiac recovery was less remodeled than in patients without cardiac recovery. Therefore, we confirmed that the remodeling of microstructures is a biomarker of cardiac recovery. This finding will help adjust clinical therapy of HF patients and avoid unnecessary transplantations in HF patients likely to recover their cardiac function after LVAD unloading.

Index Terms—Left Ventricle Assist Device (LVAD), Ryanodine Receptor 2 (RyR2), Junctophilin 2 (JPH2), Microstructure, Molecular remodeling, Heart Failure (HF), Cardiac recovery

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I. INTRODUCTION

Left ventricular assist devices (LVADs) are mechanical pumps that can be implanted in heart failure (HF) patients as a bridge-to-transplantation. The LVAD pumps blood from the left ventricle into the aorta (Fig. 1), which then sends the blood to the body. Therefore, the LVAD replaces the heart as blood pump. In some cases, patients respond to LVAD implantation with sustained cardiac recovery, and they no longer need a heart transplant [1– 3]. Because of left ventricular recovery, several studies investigated the recovery of myocardial function at the molecular level and have identified the molecular process resulting in contractile force as a point of interest [4].

Contractile force in the heart is generated by muscle cells (cardiomyocytes) that are composed of several proteins that are necessary for proper intracellular ion signaling. The main ion type for signaling is calcium [5]. Calcium enters cardiomyocytes through calcium channels in the sarcolemma (Fig. 2), which forms regular invaginations that insert between myofibrils, termed

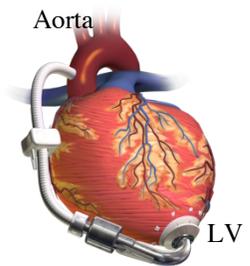


Fig. 1. Heart with LVAD. Connected from the left ventricle to the aorta (Courtesy of ReliantHeart, Inc.).

84112 and ²Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, Salt Lake City, UT 84112, USA. (e-mail: yjee328@gmail.com).

transverse tubules (t-tubules) (Fig. 2). T-tubules are present exclusively in striated muscle [6]. Calcium then activates RyR2 (ryanodine receptor 2) (Fig. 2), which releases a significant amount of calcium from the sarcoplasmic reticulum (SR) (Fig. 2) [7]. Then, it triggers the sarcomere (Fig. 2), which is a basic unit

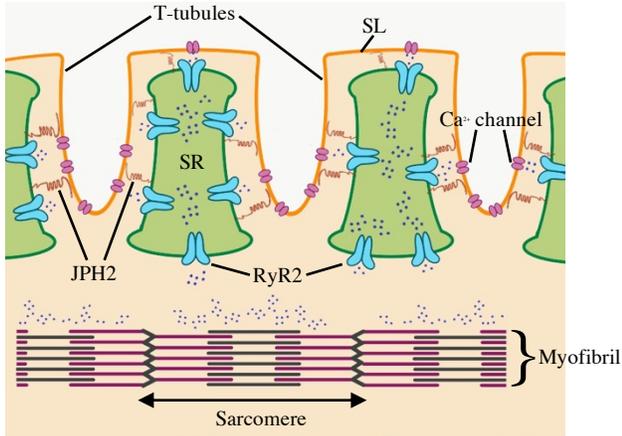


Fig. 2. T-tubules, RyR2, JPH2, SR, calcium channels, sarcolemma (SL), sarcomere, myofibril, calcium channels in cardiomyocytes.

of the striated muscle tissue, to contract itself. JPH2 (junctophilin2) (Fig. 2) is a junctional protein between the sarcolemma as well as t-tubules and the SR [8]. Therefore, the t-tubule system (t-system), JPH2, and RyR2 are essential microstructural features for a proper excitation-contraction process.

However, in HF patients, the excitation-contraction process malfunctions and leads to functional and structural cellular remodeling [9]. Seidel *et al.* [9] found that the t-system is remodeled in HF into a sheet-like shape. Also, several studies have found that the t-system remodeling, including the t-sheet like remodeling and low density of the t-system [9] [10] were co-related with failing contraction of the ventricles [11]. Additionally, developing predictive biomarkers for the ventricular failure [12] [13] has been researched, but results are not conclusive. Furthermore, establishing and validating a biomarker (predictor) of cardiac recovery after LVAD unloading by observing RyR2 [9] and JPH2 [14] localization changes relative to the remodeled t-system have not yet been sufficiently studied.

Assuming that proper localization of JPH2 and RyR2 to the t-system is crucial for normal cardiomyocyte function, we hypothesize that only

patients without remodeling of microstructural features can recover their cardiac function. To do this, we will apply 3-dimensional (3D) microscopy to human HF tissues to observe the changes in microstructural features and compare RyR2 and JPH2 localization in healthy donor tissues, HF tissues, and HF tissues with cardiac recovery to find a biomarker of cardiac recovery after LVAD unloading.

If remodeling of microstructural features is found to be a biomarker of cardiac recovery, the predictor can help reduce morbidity of long-term use of a LVAD and unnecessary transplantations in HF patients likely to recover their cardiac function after LVAD unloading [10]. Also, a better understanding of functional and structural remodeling will contribute to developing innovative therapies for HF.

II. METHODS

To better understand left ventricular tissue's functional and structural remodeling in HF without cardiac recovery, we obtained tissues that patients with recorded left ventricular ejection fraction before LVAD implant (LVEF-preLVAD) and after LVAD implant (LVEF-postLVAD). Also, we measured the left ventricular end diastolic diameter before LVAD implant (LVEDD-preLVAD) and after LVAD implant (LVEDD-postLVAD).

A. Tissue Acquisition

In this study, all tissues were collected at the University of Utah with approval of the Institutional Review Board (IRB). Left ventricular tissues were collected retrospectively from 8 donor tissues as well as 10 HF tissues with and 5 without recovery after LVAD unloading. Donor tissues were from non-heart failure hearts that could not be transplanted. HF tissues were from patients who did not recover after LVAD unloading and were retrieved when LVAD implantation was in progress. The HF tissues with cardiac recovery after LVAD implant were from patients who recovered favorable to LVAD unloading and were obtained when the LVAD was implanted. The tissue samples were snap-frozen in an optimal cutting temperature compound (Tissue-

Tek® O.C.T.™ Compound, Sakura® Finek, Torrance, California).

B. Tissue Processing

We sectioned the frozen tissues with 100 μm thickness using a cryostat microtome (CM1950, Leica Biosystems, Nussloch, Germany). The cardiac tissue sections were then labeled with DAPI (4',6-diamidino-2-phenylindole) stain (Alexa Fluor-405 conjugate, Thermo Fisher Scientific Inc, Göteborg, Sweden) for cell nuclei. WGA (Wheat germ agglutinin) stain (Alexa Fluor-561 conjugate, Thermo Fisher Scientific Inc) was used to label the extracellular matrix and t-system. RyR2 and JPH2 were labeled with RyR2 (C3-33, Thermo Fisher Scientific Inc) and JPH2 (ab110056, Thermo Fisher Scientific Inc) fluorescent primary antibodies (mouse), followed by secondary antibodies that are Alexa Fluor® 488 and Alexa Fluor® 633 goat anti-mouse secondary antibodies (Thermo Fisher Scientific Inc), respectively. Then, we mounted on a cover glass, and embedded in Fluoromount-G™ (Thermo Fisher Scientific Inc).

C. 3D Confocal Microscopy

We imaged the mounted tissue sections *via* a confocal microscope (Leica TCS SP8, Leica Biosystems, Nussloch, Germany) equipped with a 63 \times oil immersion lens. A 1 AU pinhole size with 0.1 \times 0.1 \times 0.1 μm^3 voxel size were set. To improve signal-to-noise ratios at high imaging depths, power of the lasers was linearly increased with increasing distance from the tissue surface. We restricted the depth of imaging stacks to be 25 μm in order for preventing from a decrease of signal-to-noise ratios at larger depths.

D. Image Processing and Analysis

We improved image qualities before analyzing by computational process. The 3-D image stacks were deconvolved by the Richardson-Lucy algorithm (REF) using measured point spread functions. Also, we corrected depth-dependent signal attenuation. Images of nuclei were segmented using a histogram-based threshold of mode + 2 standard deviations (SD) [9]. The volume covered by the nuclei segments was excluded from further analysis in order to remove artifacts caused by unspecific antibody

labeling. Myocytes in the image stacks were segmented using a water-shed approach and manual merging. The images of myocytes were used for a distance transform [15]. RyR2 and JPH2 images were segmented with a threshold of mode + 2 SD. RyR2 and JPH2 segments were then used to calculate intensity distribution and intensity-weighted average distance of RyR2 and JPH2 clusters, respectively, from the sarcolemma [9]. The regions where RyR2 and WGA overlap were excluded in this calculation. We performed all processing and analyses with MATLAB R2018a (MathWorks Inc., Natick, Massachusetts).

E. Statistical Analysis

By using RyR2 and JPH2 segments obtained in D section, we calculated the distance between sarcolemma and RyR2 and JPH2, respectively. The statistical data are presented as mean \pm standard error (SE). One-way ANOVA with Bonferroni-Holm correction for multiple comparisons was applied when testing for differences in distribution of RyR2 and JPH2, respectively [9]. Differences were considered significant *, **, and *** if $p < 0.05$, $p < 0.01$, and $p < 0.001$.

III. RESULTS

A. Clinical Measurements and Classification of Patients

The study population was comprised of 8 donors that were the controls in our study, 10 HF patients without cardiac recovery, and 5 HF patients with cardiac recovery. We classified HF without and with cardiac recovery by LVED and LVEF. HF patients without cardiac recovery were defined when LVEF-post LVAD is less than 35% and the relative improvement of LVEF is less than 50 % regardless of LVEDD. Responders (RS) included in HF patients with cardiac recovery were defined when LVEF-post LVAD was greater than 40% and LVEDD-post LVAD was less than 6cm.

B. Remodeling of the t-tubules to t-sheets in HF cardiomyocytes

We obtained left ventricular tissues for histological

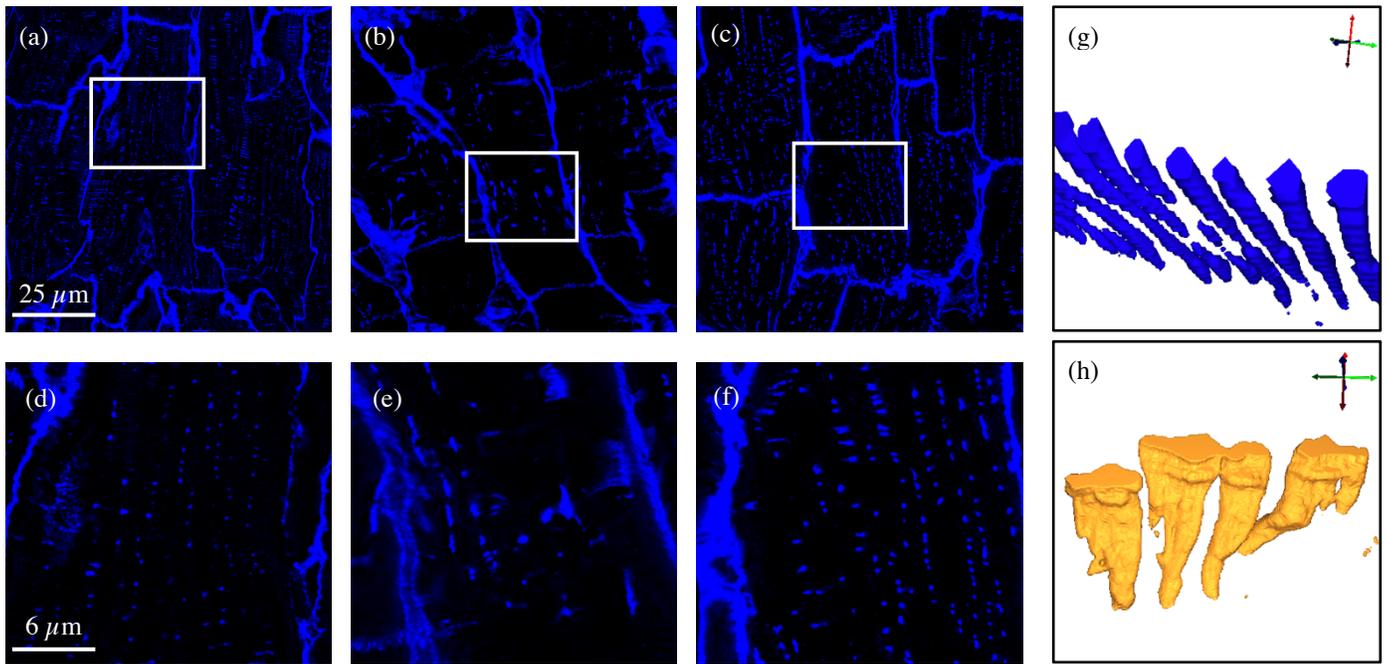


Fig. 3. Two-dimensional image of WGA-labeled left ventricular tissue obtained from a control, HF patient without cardiac recovery and with cardiac recovery at the time of LVAD implantation and three-dimensional reconstruction of t-system from control and HF patients without cardiac recovery. The size of images was $102.4 \times 102.4 \mu\text{m}$: (a) is an image from control tissue. (b) is an image from HF without cardiac recovery, and (c) is an image from HF with cardiac recovery. Magnified images from boxed region in (a), (b), and (c) are (d), (e), and (f), respectively. (g) is three-dimensional tubular invaginations (t-tubules) of the sarcolemma from control. (h) is three-dimensional remodeled t-system component from HF without cardiac recovery. Scale bar of (a) that applies to (b) and (c) is $25\mu\text{m}$. Scale bar of (d) that applies to (e) and (f) is $6\mu\text{m}$.

analysis by the confocal microscope at the time of LVAD implantation. Two-dimensional images of the control (Fig. 3 (a) and (d)) and HF with cardiac recovery (Fig. 3 (c) and (f)) exhibited dense and striated t-tubules. However, two-dimensional images of HF without recovery exhibited sparse and irregular remodeled t-systems into a sheet shape (t-sheets) (Fig. 3 (b) and (e)).

Also, the longitudinal components of the t-sheets parallel to the main axis of the cardiomyocytes were visible in Fig. 3 (b) and (e). We also visualized 3D images that exhibited the tubular t-system in the controls (Fig. 3 (g)) while HF without cardiac recovery exhibited sheet-like remodeling (Fig. 3 (h)).

C. RyR2-Sarcolemma Distance and Myocardial Functions

We found RyR2 localization changed in HF without recovery (Fig. 4 (b) and (e)). RyR2 in the control (Fig. 4 (a) and (d)) and RS (Fig. 4 (c) and (f)) exhibited regular distribution and co-localized with t-tubules while many of the RyR2's were clustered in the HF-without-cardiac-recovery tissue. Also, most RyR2 clusters were redistributed with

increasing distance from the sarcolemma in HF without cardiac recovery as compared to the means of distance of the RyR2 from the sarcolemma in the controls and HF with cardiac recovery (0.69 ± 0.032 versus 0.48 ± 0.032 and 0.62 ± 0.02 , respectively). The means of HF without cardiac recovery and controls were significantly different; $p < 0.001$ (Fig. 4 (g)). However, the mean of HF without cardiac recovery and RS were not significantly different ($p = 0.22$). Thus, the distance of RyR2 from the sarcolemma was associated with cardiac function.

D. JPH2-Sarcolemma Distance and Myocardial Function

We found that JPH2 localization changed in HF without recovery (Fig. 4 (b) and (e)) exhibited a similar distribution to RyR2 localization changes in HF without recovery. JPH2 in the controls (Fig. 4 (a) and (d)) and RS (Fig. 4 (c) and (f)) were striated and were located proximal to the sarcolemma while the JPH2's were clustered in HF without cardiac recovery (Fig. 4 (b) and (e)). Additionally, most JPH2 clusters in HF without cardiac recovery were

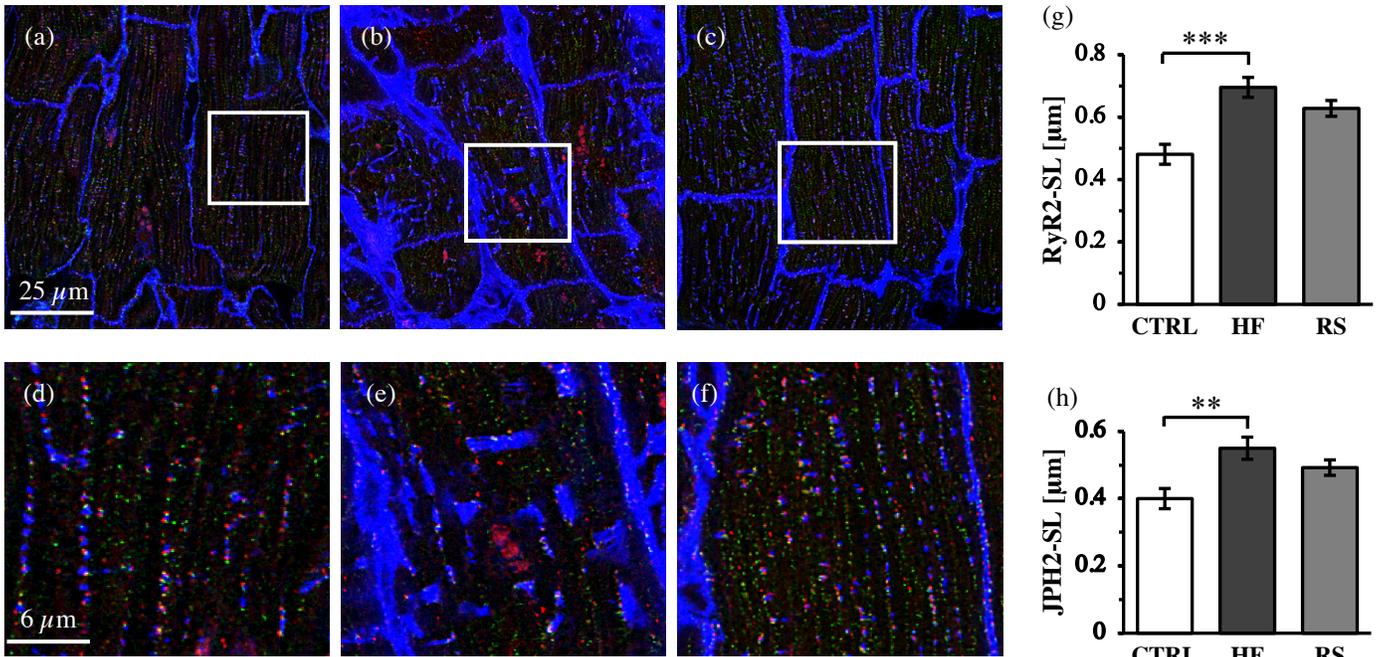


Fig. 4. Two-dimensional image of WGA (blue), RyR2 (green), and JPH2 (red) - labeled left ventricular tissue obtained from controls, HF patients without cardiac recovery and with cardiac recovery at the time of LVAD implantation and analyses of RyR2-Sarcolemma (SL) distance or JPH2-SL distance. The size of the images was $102.4 \times 102.4 \mu\text{m}^2$. Image (a) is of the control tissue. Image (b) is of HF without cardiac recovery, and (c) is an image from HF with cardiac recovery. Images (d), (e), and (f) are magnified images from the boxed region in (a), (b), and (c), respectively. Image (g) is the RyR2-SL distance (mean \pm SE) compared between control (CTRL), HF without cardiac recovery (HF) and HF with cardiac recovery (RS). (h) is JPH2-SL distance (mean \pm SE) compared between control, HF and RS. ** significant at $p < 0.01$ and *** significant at $p < 0.001$. Scale bar of (a) that applies to (b) and (c) is $25 \mu\text{m}$. Scale bar of (d) that applies to (e) and (f) is $6 \mu\text{m}$.

located farther from the sarcolemma than in the than in the controls and RS (Fig. 4 (h)). The mean distance of JPH2 from the sarcolemma in HF without cardiac recovery was greater than the mean in the controls (0.55 ± 0.033 versus 0.40 ± 0.03). Two groups of controls and HF without cardiac recovery were significantly different: $p < 0.01$. The mean of RS was 0.492 ± 0.023 , which was greater than the mean in the controls but less than the mean of HF without cardiac recovery. However, the mean of RS was not significantly different than the mean in HF without cardiac recovery ($p = 0.26$). Therefore, the distance of JPH2 from the sarcolemma was also associated with cardiac function.

E. Volume Fraction of WGA and Correlation between RyR2 and JPH2

We explored the volume fraction (VF) of WGA in controls, HF, and RS tissues. VF of WGA in HF showed a higher value than the controls and RS tissues (23.6 ± 0.84 versus 21.25 ± 0.74 and 22.8 ± 0.68 , respectively), and the mean of control and the

mean of HF without cardiac recovery were significantly different ($p < 0.05$) (Fig. 5 (a)). We also investigated the correlation between RyR2 and JPH2. We found that the controls and RS groups had greater distances between RyR2 and JPH2 than the HF group (0.182 ± 0.014 and 0.205 ± 0.025 versus 0.141 ± 0.015). The mean distance between RyR2 and JPH2 in HF without cardiac recovery was significantly different than the mean of RS ($p < 0.05$) (Fig. 5 (b)).

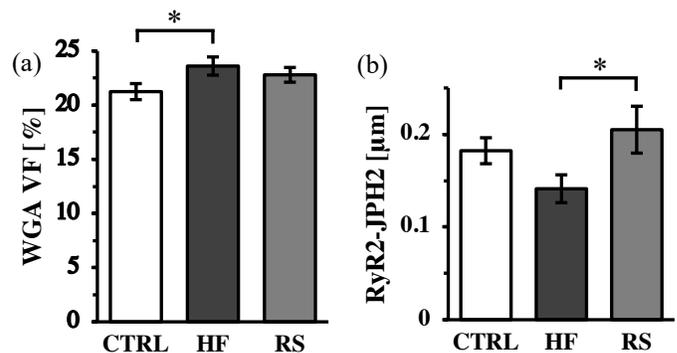


Fig. 5. Statistical analysis of image data. (a) is the volume fraction (VF) of WGA (mean \pm SE) in CTRL, HF and RS. The correlation between RyR2 and JPH2 is shown in (b) (mean \pm SE). * significant at $p < 0.05$

IV. DISCUSSIONS

End-stage HF is a complex clinical syndrome caused by structural or functional disorders of the heart [16]. HF is a major disease and expensive healthcare expenditure with a prevalence of over 5.8 million in the US [16] [17]. End-stage HF patients often need a heart transplant surgery, but due to donor heart shortages, LVAD is often implanted for end-stage HF patients as a destination therapy, or an occupational therapy, as well as bridge-to-transplant therapy [18] [19]. Patients being bridged with an LVAD has increased in the last decade [20–23], and the LVAD improved outcomes and provided a reasonable quality of life for HF patients [24]. As interest in LVAD recovery cases increases, we hypothesized that there could be microstructural differences between a group of HF without cardiac recovery and an RS group. Specifically, we hypothesized that only patients without remodeling of the microstructural features can recover their cardiac function. To evaluate RyR2 and JPH2 as biomarkers of cardiac recovery for LVAD patients, we investigated tissues with confocal microscopy and a computational approach.

A. T-system Remodeling in HF Tissues

Many studies showed that t-tubules in HF tissues were disorganized and their density was reduced [9] [25–28]. We observed and compared the t-system in HF *versus* control. We validated our t-system findings to the microscopic approach of T. Seidel *et al* [9]. Our three-dimensional imaging of the left ventricular apical core tissues from LVAD patients confirmed that the t-systems is more sheet-like than tubular (Fig. 3 (b), (e), and (h)). The remodeling of the t-system, referred to as t-sheets in a previous study [9]. Also, the t-sheets in HF tissues were more irregular and sparser than t-tubules in both control tissues and RS tissues (Fig. 3 (d) and 3 (e)). Dilation of t-system resulted in the longitudinal sheets parallel to the long axis of the cardiomyocytes with low density. These remodeling in HF tissues were related to increasing distances between RyR2, JPH2, and the sarcolemma. However, measuring circularity of t-tubules and t-sheets would better support the above findings. E. Page *et al* reported that t-tubule remodeling in HF caused disorders of Excitation-

Contraction (EC) coupling that is required for normal cardiac signaling [29]. Therefore, we concluded that the mechanistic disorder and the t-system remodeling in HF tissues are associated with HF disease.

B. Increased Distance of RyR2 and JPH2 from Sarcolemma in HF Tissues

Intensity distribution and intensity-weighted average distance of RyR2 and JPH2 from the sarcolemma were calculated by segmenting RyR2 and JPH2 into 3D images. RyR2 localizations in the control and HF tissues exhibited similarity to JPH2 localizations and were more clustered. We observed that RyR2 and JPH2 were located proximal to the sarcolemma in the control tissues whereas RyR2 and JPH2 clusters in the control tissues were re-localized to the sarcolemma from the t-system (Fig. 4 (d), (e), and (f)). We confirmed the changes of RyR2 and JPH2 localization in HF tissues using statistical analyses. Most RyR2 and JPH2 clusters were redistributed with increasing distance from the sarcolemma in HF without cardiac recovery (Fig. 4 (g) and (h)). The localization differences of RyR2 and JPH2 in between HF tissues and control were significantly different ($p = 0.001$ for RyR2 and 0.005 for JPH2). We also checked the distance between RyR2 and JPH2 to observe correlations. RyR2 and JPH2 in HF tissues tended to be more clustered and disorganized than in control tissues. We analyzed this statistically to calculate the significance level but the control versus the HF groups were not significantly different ($p = 0.22$ for RyR2 and 0.26 for JPH2). Overall, we concluded that groups with better cardiac function showed an increased distance level between RyR2 and JPH2. Our conclusion is consistent with JPH2 and RyR2 studies. They reported that mutation of JPH2 in cardiomyocytes causes hypertrophic cardiomyopathy in humans [14] [30–31]. Also, several studies showed that dysfunctional RyR2 causes delayed depolarization during EC coupling, leading to HF diseases [32–35]. However, these studies showed cardiac mechanistic changes from the disordering of JPH2. We showed HF remodeling of JPH2 with microscopic methods, and we expected that the distances between RyR2 and JPH2 or distances from the sarcolemma to RyR2 or JPH2 would be

biomarkers for cardiac recovery. Further studies to determine appropriate criteria between cardiac recovery and HF for our findings should be completed to be used by clinicians as biomarkers of cardiac recovery in HF patients.

C. Extracellular Matrix Remodeling in HF Tissues

Through computational analyses, we observed that the VF of extracellular matrix labeled with WGA in cardiomyocytes from HF tissues was greater than in the control tissues, showing a statically significant different (p-value). (Fig. 5 (a)). This indicates that the extracellular matrix in HF was remodeled as well as the t-system in HF tissues. Our result is in an agreement with well-known fact that severe fibrosis in cardiomyocytes leads to failing cardiac function.

D. Restoration of Microstructural Features in RS Tissues

We observed that the t-system in HF patients with cardiac recovery (RS) tissues exhibited similarities to the t-system in control tissues. Most t-systems in RS tissues were tubular like the control tissues, and t-sheets were rare (Fig. 3 (a), (e), (d), and (f)). RyR2 and JPH2 in RS tissues also exhibited similarities to the control tissues. The distance of RyR2-sarcolemma and JPH2-sarcolemma in RS tissues exhibited a lower value than in HF tissues, and this result was similar to the control. However, unlike results compared among the control and HF groups, we did not observe a significant difference between the means of distance between RyR2 and the sarcolemma or JPH2 and the sarcolemma of RS and HF groups (Fig. 4 (g) and (h)). Although our research did not show significant differences between HF and RS groups, the overall tendency of RS groups was similar to the control in our findings. The RyR2-sarcolemma distances in the RS were greater than in HF, and the JPH2-sarcolemma distances were also greater in RS. The correlation between RyR2 and JPH2 was also higher in RS than in HF (Fig. 5 (b)). Future work should examine the similarities in the t-system and RyR2 and JPH2 localizations among the control and RS tissues to represent restoration of microstructural features. However, our findings are still promising because it adds more findings to the area of cardiac recovery with a microscopy approach.

Because JPH2, RyR2, and t-tubules are essential microstructural proteins for the calcium diffusion that allows human hearts to work normally [5–8], we conclude that healthy phenotypes of microstructural features at the time of LVAD implantation are necessary for recovering their cardiac function after LVAD explant. The primary limitation of this study as the small number of tissue samples from patients (donor tissues (n=8), HF tissues with (n=10), and without recovery (n=5)). We expect that gathering more data from more HF patients with cardiac recovery after LVAD unloading would help present distinctive results and significant differences between HF and RS groups.

E. Clinical Perspective

Our findings that RS tissues at the time of LVAD implantation exhibited similarities to control tissues could help clinicians to select patients for LVAD explant. If doctors check the phenotypes of tissues retrieved at time of LVAD implantation including their t-system, RyR2, and JPH2 as biomarkers of cardiac recovery, the number of cardiac recovery cases of LVAD explant surgery would increase. Additionally, it would be easier for clinical staff to prioritize heart transplants.

By the year 2030, it is predicted that an additional 3 million people in the U. S. will develop HF [36]. In addition to this, it was reported that the prognosis of the HF patients is poor and worse than many cancer diseases. Many HF patients, caregivers, and clinicians are not aware of the poor prognosis. Therefore, a reliable testing method like investigating the microstructures of tissues could contribute to improving the prognosis for HF patients and potentially decrease the death rate of HF patients. We expect that our findings could help in developing this testing method in the future.

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