Pharmaceutical interventions in a murine cardiac aging model.

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Abstract

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Heart disease is the number one killer in developed and undeveloped nations, with aging being the number one factor contributing to its diagnosis. Currently, there are no preventative medications to prevent this pathology, especially the increasingly common disorder heart failure with preserved ejection fraction (HF/pEF). Two therapeutics that might be good preventative medications are rapamycin, an inhibitor of the target of rapamycin pathway that has been shown to extend lifespan, and the tetra-peptide SS-31. Using a model of murine cardiac aging, two experiments were designed to investigate if rapamycin and SS-31 could improve heart function. Using echocardiography, qPCR, and western blots, we found that in both conditions, there appears to be no improvement in functional changes. Electron microscopy did show beneficial changes in mitochondrial and cytoskeletal ultrastructure. Additionally, while there are many proteomic changes by mass spectroscopy, these changes were difficult to identify in qPCR or western blot analysis. However, in the SS-31 persistence experiment, a large morphological improvement in the hearts, suggested a positive and persistent benefit pf the peptide. While taking the first step in the right direction, future work will need to expand mouse numbers and parameters measured by qPCR and western blot to better understand what is occurring with these therapies and whether they would be good for humans as a preventative measure for heart disease.

I. Introduction

In 2012, both the Center for Disease Control in the US, and the World Health Organization marked heart disease as the number one killer. Currently, heart failure with preserved ejection fraction (HF/pEF) represents about 50% of heart failure cases and affects more women than men (Oghlakian et al., 2011; Udelson, 2011). In heart failure with preserved ejection fraction, the left ventricle decreases in compliance, while increasing pressure (Oghlakian et al., 2011; Udelson, 2011). Outside of heart failure conditions, as individual's age, the heart experiences a host of changes. These include increased hypertrophy, systolic and diastolic time intervals, and diastolic dysfunction (Oghlakian et al., 2011; Udelson, 2011). These conditions can be measured by left ventricular mass index (LVMI), myocardial performance index (MPI), and tissue Doppler imaging (TDI), respectively. LVMI measures the mass of the heart, and increases in age and cardiac dysfunction (Gladden et al., 2014). MPI is measures systolic and diastolic time intervals to express overall systolic and diastolic ventricular function, time that increases as the mice age (Gladden et al., 2014). TDI is used to measure ventricular filling velocities at the mitral valve. As diastolic function becomes impaired with age, the early component of filling, designated as E' or Ea, related to passive ventricular relaxation, is impaired, and the later component of filling, related to left atrial contraction and designated A' or Aa, becomes more dominant (Gladden et al., 2014). Thus, during aging the ration of E'/A' (or Ea/Aa) declines. Mice experience these same aging heart characteristics, making them good model organisms to study (Chiao and Rabinovitch, 2015; Dai and Rabinovitch, 2009). Currently, we have no preventative medication to attenuate this pathology (Gladden et al., 2014). Some of the greatest successes in preventing cardiac mortality have relied on the use of drugs and surgeries post-cardiac symptoms, however, individuals with only HF/pEF do not experience systolic failure, may not have suffered from coronary disease or myocardial infarction, and strategies to reduce atherogenesis and cardiac workload are not effective to prevent HF/pEF (Gladden et al., 2014). Recent work in our laboratory, however, has suggested the use of SS-31 (clinical name: Bendavia) and rapamycin as preventative compounds in aging hearts to maintain function, including improved diastolic function.

SS peptides, named after co-inventors Drs. Peter Schiller and Hazel Szeto, are a group of tetra-peptide compounds, designed initially as synthetic opioid pain killers, but serendipitously found to be good mitochondrial antioxidants (Szeto and Birk, 2014). SS peptides have two aromatic amino acids alternating with two basic amino acids, giving a positive charge (Szeto and Birk, 2014). In SS-31, the amino acid order is D-Arg-Dmt-Lys-Phe-NH_2 (Szeto and Birk, 2014). SS-31 enters the inner mitochondrial membrane via passive diffusion. The current understanding of the peptides interaction is that it binds to cardiolipin, imparting enhanced cytochrome C-mediated electron transfer and ATP production, and reduced cytochrome C peroxidase activity (Birk et al., 2014; Szeto and Birk, 2014). SS-31 is also known to have antioxidant effects, but this is not now believed to be its main role in mitochondria. Already, SS-31 has shown an improvement in decreasing infarct size in cardiac ischemia/reperfusion injury, preventing renal ischemia reperfusion injury and fibrosis, reducing skeletal muscle insulin resistance in mice fed a high fat diet, and reducing the MPTP-induced Parkinson mice pathology (Dai et al., 2013; Eirin et al., 2014; Kloner et al., 2012; Liu et al., 2014; Sun et al., 2015; Wu et al., 2015). Currently, Bendavia is in human Phase II trials in human heart disease (NCT01572909), sarcopenia (NCT02245620), mitochondrial

myopathy7 (NCT02367014), and diabetic macular edema and age-related macular degeneration (NCT02314299).

Rapamycin is an inhibitor of mTOR (mechanistic target of rapamycin) that recapitulates many of the same effects as dietary restriction (Lamming et al., 2013; Li et al., 2014; Speakman and Mitchell, 2011; Sung and Dyck, 2012). Rapamycin or related synthetic analogs is given clinically as an immune suppressant, but work in model organisms has highlighted its role in extending lifespan and health span (Flynn et al., 2013; Lamming et al., 2013; Li et al., 2014; Wilkinson et al., 2012; Wu et al., 2013; Yang and Ming, 2012). In the Rabinovitch lab, work has shown a change in cardiac proteomics and metabolomics of old treated mice that become more similar to those of a young mouse, accompanied by improved functional performance, including reversal of diastolic dysfunction (Dai et al., 2014). Cardiac benefits have been reported by other laboratories (Flynn et al., 2013; Yang and Ming, 2012). Many studies are underway on the role of rapamycin in persisting in the body, its role throughout a life time, and varying the concentration of the compound (Flynn et al., 2013; Wilkinson et al., 2012).

With this information, we formed two hypotheses. The first hypothesis pertains to the combined therapy of SS-31 and rapamycin, and we hypothesized that SS-31 and rapamycin combined treatment would have additive benefits due to the different pathways they work in and the positive benefits they each exhibit alone. For the second study, we hypothesize that the SS-31 will have some persistence in the system since the proteomic changes we've see in the past will take time to revert to the old profile.

In the following sections, these therapies are investigated for their combined effects due to the fact that they affect different cell pathways, and the possibility of persistence of health or cellular effects even after the drug, SS-31, had been withdrawn.

II. Combined treatment with Rapamycin and SS-31

I. Functional Assessments

The most practical and clinically important functional heart assessment comes in the form of echocardiograms. As briefly described in the introduction, echo cardiograms, depending on the measurement taken, give insight into the function and health of the heart and this can be performed longitudinally over an extend period of time. Previous work in the Rabinovitch lab has shown an improvement in TDI and E/A ratios in mice treated with rapamycin or SS-31. Rapamycin and SS-31 improvements, especially in diastolic function and MPI are both gradual in onset over the 8-10 week study periods observed. In this study, mice were obtained from the National Institute of Aging Charles River colony. Both genders were studied. Previous data that showed the effects of SS-31 were largely studied in male mice, whereas previous work with rapamycin was largely performed with female mice, due to the fact that rapamycin has a much larger effect in this sex (Harrison et al., 2009). Mice were housed at 20^{O}C in an AAALAC accredited facility under Institutional Animal Care and use Committee (IACUC) supervision.

Mice were assigned into groups to equalize initial group weights and were then fed with either control diet, or diet with microencapsulated rapamycin at 2.24mg/kg/day (1xRapa, Rapamycin Holdings, San Antonio TX female) or 6.72mg/kg/day (3xRapa, Rapamycin Holdings, San Antonio TX male) and maintained on their corresponding diet for 8 weeks. Echocardiography was performed under 0.5%-1% isoflurane at baseline, 4, and 8 weeks following treatments for females, and removing the 4 week time point for males, using a Siemens Acuson CV-70 equipped with a 13MHz probe.

Experiments were performed in one cohort of female mice and two cohorts of male mice. Beginning with the female mice, there is no significant change in ejection fraction at any time point or compared to any condition in an unpaired t-test as can be seen in Figure 1. In figure 2, the female mice show a significant decrease in ejection fraction in the control group from baseline to four weeks post treatment that is significant by a paired t-test, but no other significant changes were observed. In figure 3, data is shown pertaining to the state of the left ventricle. No change is observed in the left ventricular mass index by unpaired t-test between groups, nor is there significant change overtime within groups by paired t-test. Figure 4 and figure 5 relate to components our lab has most often seen change during the treatment process with rapamycin or SS-31. In figure 4, myocardial performance index is evaluated. In a paired comparison, there is a significant increase in MPI in the rapamycin + SS-31 treatment group between the baseline measurement and the eight week time point. Additionally, in unpaired t-tests, there is a significant change between the four week control group and the four week rapamycin treated group and the four week control group and the four week rapamycin + SS-31 treated group. By eight weeks, it is possible to only see a significant change between the 8 week control group and the 8 week rapamycin group. In figure 5 we investigate the Ea/Aa ratio. In paired t-test, we observe a significant improvement in the Ea/Aa ratio from baseline to 4 weeks, and from baseline to 8 weeks in the rapamycin + SS-31 treated group. No other group shows this improvement over time. However, in unpaired t-test, we see a significant improvement in the Ea/Aa ratio at 4 weeks between the SS-31 treated group and the rapamycin + SS-31 treated group and an improvement between the 8 week control group and the 8 week rapamycin treated group.

In this female cohort of mice, we see some of the same changes we've observed in our lab in the past. In general, it is possible to see an increase (improvement) in the Ea/Aa (E'/A') ratio for the treatment groups that is larger than the control group, though it is not always significant. However, while we see significant changes in the MPI, these changes are in the opposite direction than what we would typically expect. In the past, after treatments, we saw a decrease in MPI, suggesting it is functioning at a higher level, similar to a young mouse, but in this cohort we see a general increase in the treated groups for their MPI, such as would be seen in the normal progression of advancing age. This conclusion is interesting, but at this time, it is unsure what exactly it means for the treatment groups. Finally, there is a slight trend towards a decrease in LVMI (left ventricular dimension), ejection fraction, and fractional shortening from the baseline to the 4 week time point, but after the initial change, it appears that the changes hold steady, which is what we would expect to see in HF/pEF, making these mice and drugs a good investigative tool for this condition.

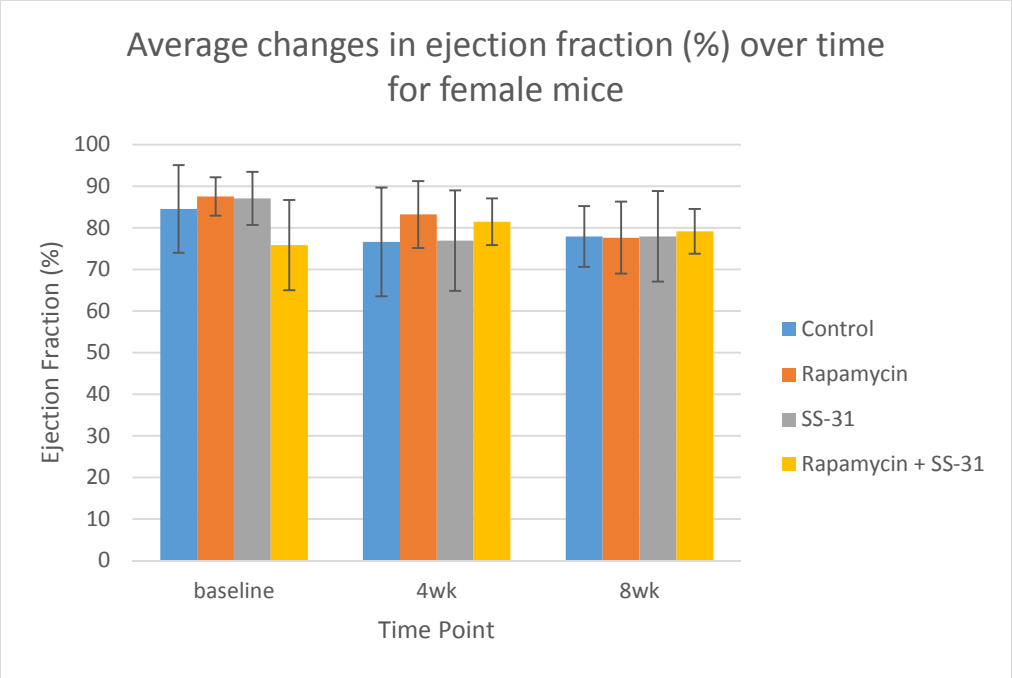


Figure 1: Measurement of ejection fraction at treatment time points from echocardiography in female mice. There is no significant change within groups across time points by paired t-test. There is also no significant difference between groups in an unpaired t-test (n=4-7). Error bars indicated standard deviation.

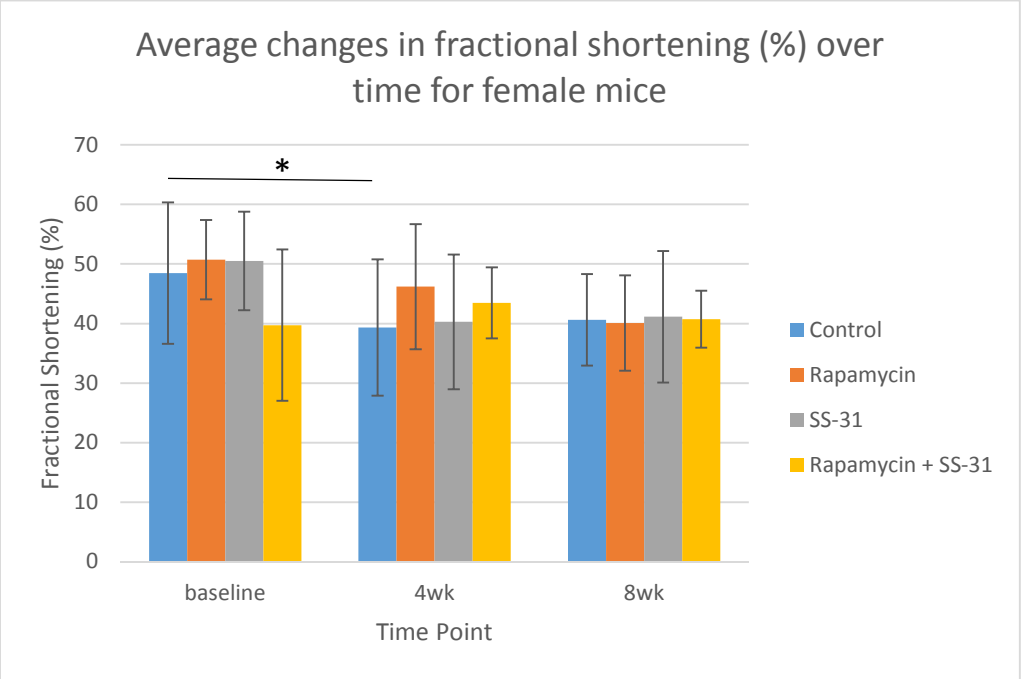


Figure 2: Measurement of changes in fractional shortening in female mice at baseline, 4 weeks, and 8 weeks after treatment. There are no significant comparisons, except between the control group at baseline and four weeks. There is a significant decrease in fractional shortening by paired t-test between the baseline control group and the four week control group (*=$p<0.05$) (n=4-7). Error bars indicated standard deviation.

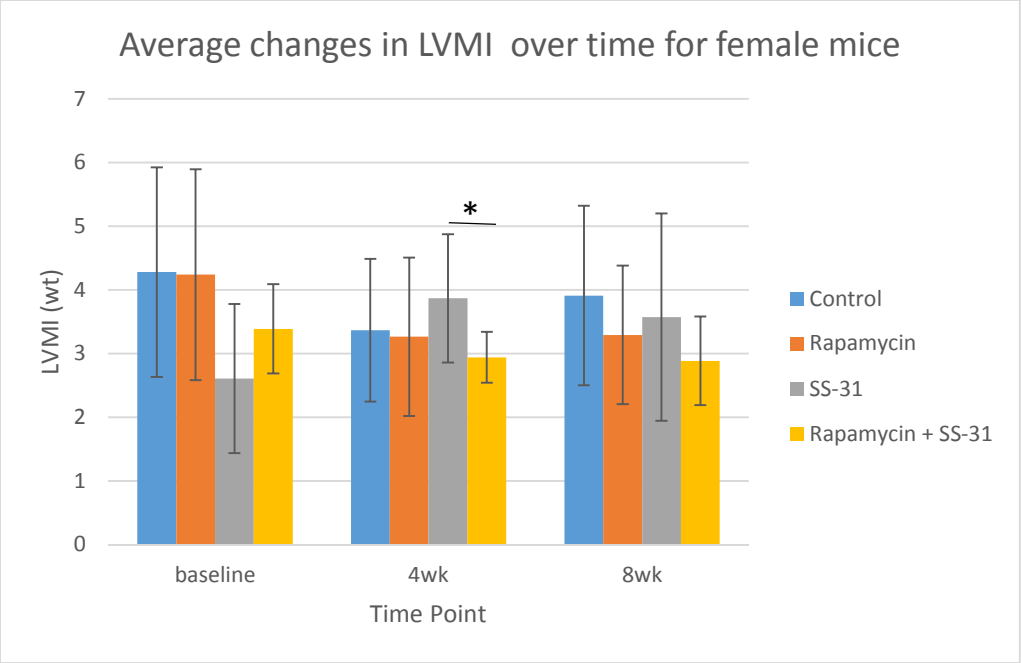


Figure 3: There was a significant decrease in LVMI between the 4 week SS-31 treated group and the rapamycin + SS-31 treated group by unpaired t-test (*=p<0.05). No other significant changes were observed between time points within groups and between groups within time points in the left ventricle mass index (n=4-7). Error bars indicated standard deviation.

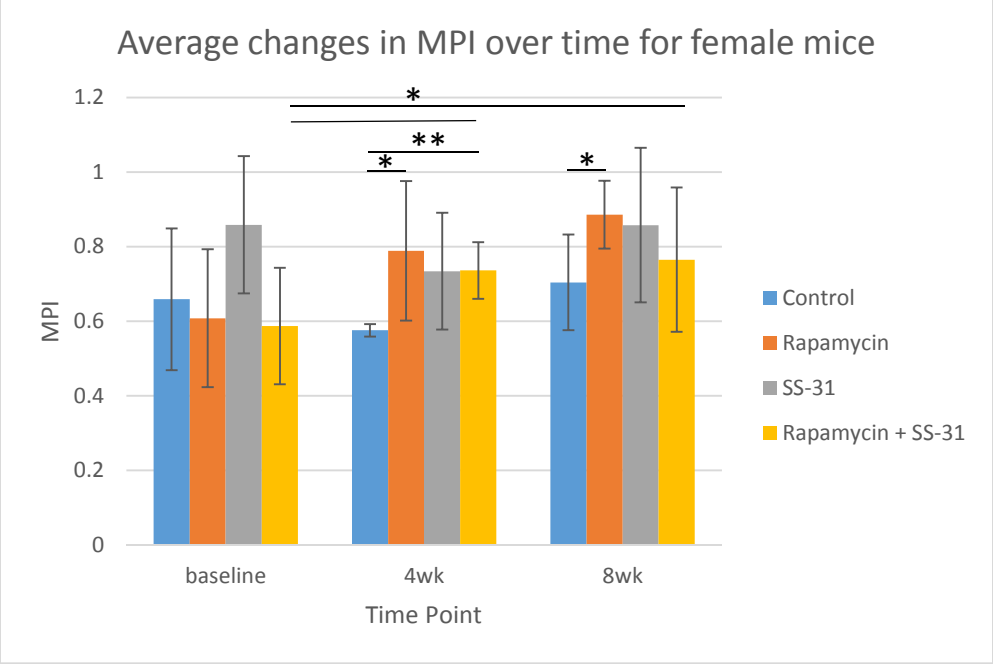


Figure 4: Significant changes in MPI are observed both within time points and across time points in female mice. The MPI is significantly increased in the rapamycin + SS-31 treated group between the baseline measurement and the 4 week time point and between baseline and the eight week time point by paired t-test (*=p<0.05). Additionally, there was a significant increase in MPI between the control group and the rapamycin group at both the 4 week time point, and the 8 week time point as measured by unpaired t-test (*=p<0.05). The largest change, however, was seen at the four week time point, where there was a significant increase in the MPI between the

control group and the rapamycin + SS-31 treated group (**=p<0.01). No other significant changes were noted (n=4-7). Error bars indicated standard deviation.

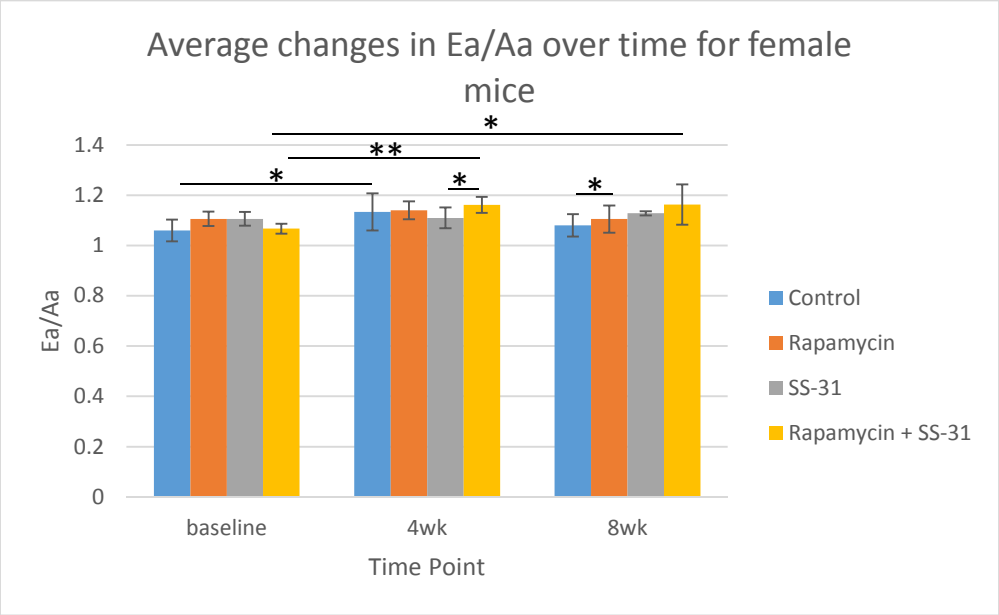


Figure 5: Measurement of E'/A' ratios was significantly changed in some conditions for female mice. In paired t-tests, there was a significant increase in E'/A' ratios between the baseline and 4 week control group, the baseline and 4 week and the baseline and 8 week time point for the rapamycin + SS-31 treated group by paired t-test (*=p<0.05, **=p<0.01). Additionally, there was a significant increase in the rapamycin + SS-31 treated group when compared to the SS-31 treated group at 4 weeks, and a significant increase in the rapamycin treated group compared to the control group at the 8 week time point by unpaired t-test (*=p<0.05). Error bars indicated standard deviation.

The two male cohorts of mice gave different results from the parameters measured. In the first cohort, AT2, the only significant change seen in ejection fraction was a decrease in the rapamycin treatment group, as assessed by a paired t-test, as can be seen in figure 6 ($p<0.05$). In contrast, the second cohort of male mice, AT4, showed a significant increase in ejection fraction from baseline to 8 weeks for the control treatment, rapamycin treatment, and rapamycin + SS-31 by unpaired t-test ($p<0.05$). In figure 7, the changes in fractional shortening are shown. In paired comparisons, AT2 control is decreased after 8 weeks, while AT4 control, rapamycin, and rapamycin + SS-31 all had significant increases after 8 weeks ($p<0.05$). Additionally, AT4 SS-31 treated mice had a significant decrease in fractional shortening compared to both rapamycin and rapamycin + SS-31 at the eight week time point by unpaired t-test ($p<0.05$). LVMI changes are shown in figure 8. There were no changes observed in AT2, but at the 8 week time point by unpaired t-test the SS-31 treated mice had a significant increase compared to the rapamycin treated group and the rapamycin + SS-31 treated group had a significant increase compared to the SS-31 group ($p<0.05$). MPI changes are shown in figure 9 below for the male cohorts of mice. MPI had no significant changes for the AT4 cohort, but there was a significant decrease from baseline to 8 weeks in the rapamycin treated group by paired t-test in the AT2 cohort ($p<0.05$). Finally, figure 10 shows the changes in the E'/A' ratio in AT2 and AT4. Again, there were almost no significant changes in the two groups, except for a significant increase from baseline to 8 weeks for the SS-31 treated mice in the AT2 cohort by paired t-test ($p<0.05$).

The changes we see with the male cohort are unlike that which we would expect from past experiments and the general pathology of HF/pEF. The male mice have few changes in MPI and E'/A' ratios, which is where most of the changes are observed in HF/pEF, while there are quite a few changes in the ejection fraction and fractional shortening, which is the opposite of what we expect. While there are many factors that affect the data, some of which are detailed below, we cannot disregard the fact that these mice might be showing a cardiac aging profile that does not match that of HF/pEF. Additionally, the ejection fraction and fractional shortening, for the most part, increased with the treatments given to the mice, suggesting that rapamycin and SS-31 alone or combined, could improve these measurements of cardiac function. The lack of significant changes make comparisons more difficult for the LVMI, MPI, and E'/A' measurements, but in general, there appears to be a trend towards improvement in MPI and E'/A' changes. In contrast, the LVMI appears to trend towards an increase, an indicator of hypertrophy, such as normally accompanies cardiac aging. Overall, these changes suggest an interesting interaction in the male mice and point out possible therapies that might be targetable that are not usually considered due to our focus on HF/pEF.

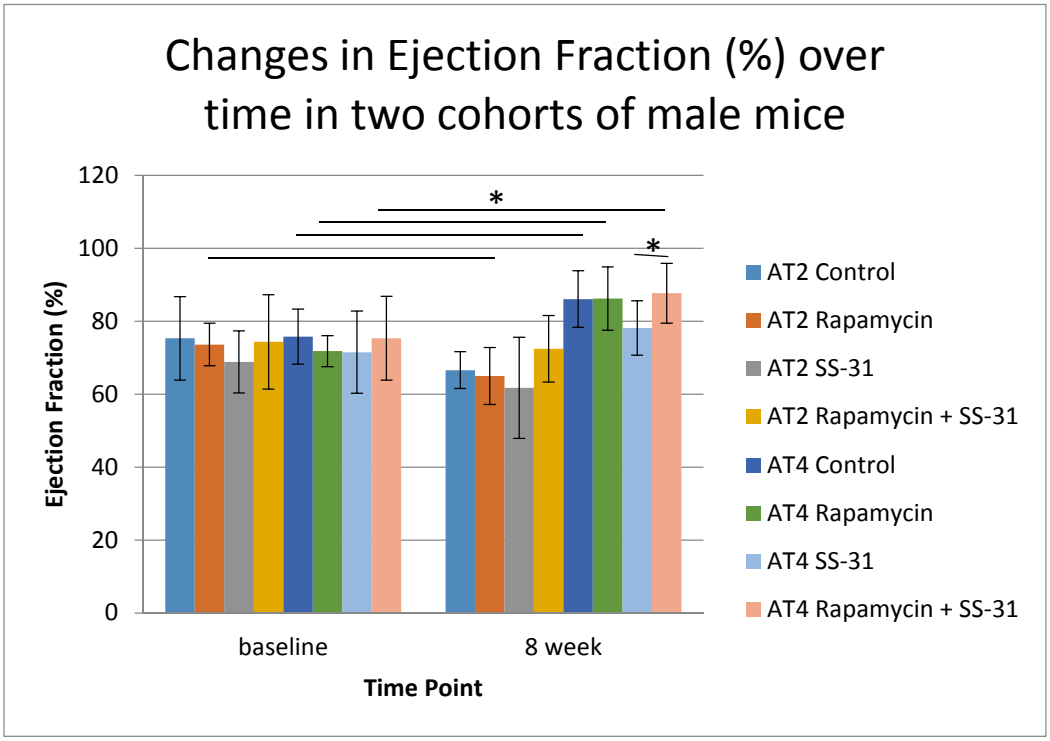


Figure 6: In the male cohorts, we see some significant changes between groups, but mostly in the second cohort of male mice were observed in ejection fraction. In the first cohort of mice, AT2, the only significant change occurred as an increase between the baseline for rapamycin and the 8 week time point for rapamycin by paired t-test (*=p<0.05). In the second cohort, AT4, there were more significant changes, including significant increases by paired t-test between baseline and the 8 week time point for the control treatment, rapamycin treatment, and rapamycin + SS-31 treatment (*=p<0.05). Additionally, in AT4, there was a significant increase between the SS-31 treated group and the rapamycin + SS-31 treated group at 8 weeks by unpaired t-test (*=P<0.05) (AT2 n=3-4; AT4 n=8-9). Error bars indicated standard deviation.

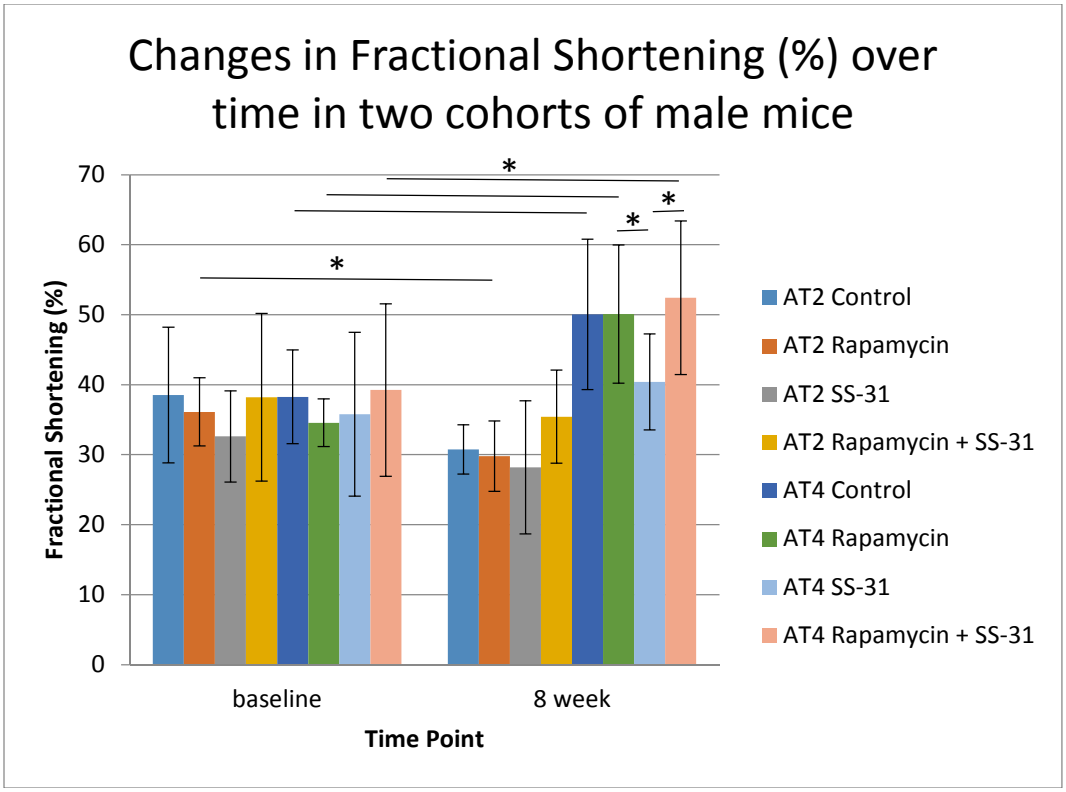


Figure 7: Changes observed in the two cohorts of male mice in their fractional shortening. In the initial cohort, AT2, there was a significant decrease in fractional shortening between the baseline and 8 week time point for the rapamycin treated mouse by paired t-test (*=$p<0.05$). In the second cohort, there was a significant increase by paired t-test between the baseline and 8 week time point for the control treated, rapamycin treated, and rapamycin + SS-31 treated mice (*=$p<0.05$). In unpaired t-tests, there was a significant decrease between the rapamycin treated and SS-31 treated group and a significant decrease between the rapamycin + SS-31 treated and SS-31 treated group at 8 weeks (*=$p<0.05$) (AT2 n=3-4; AT4 n=8-9). Error bars indicated standard deviation.

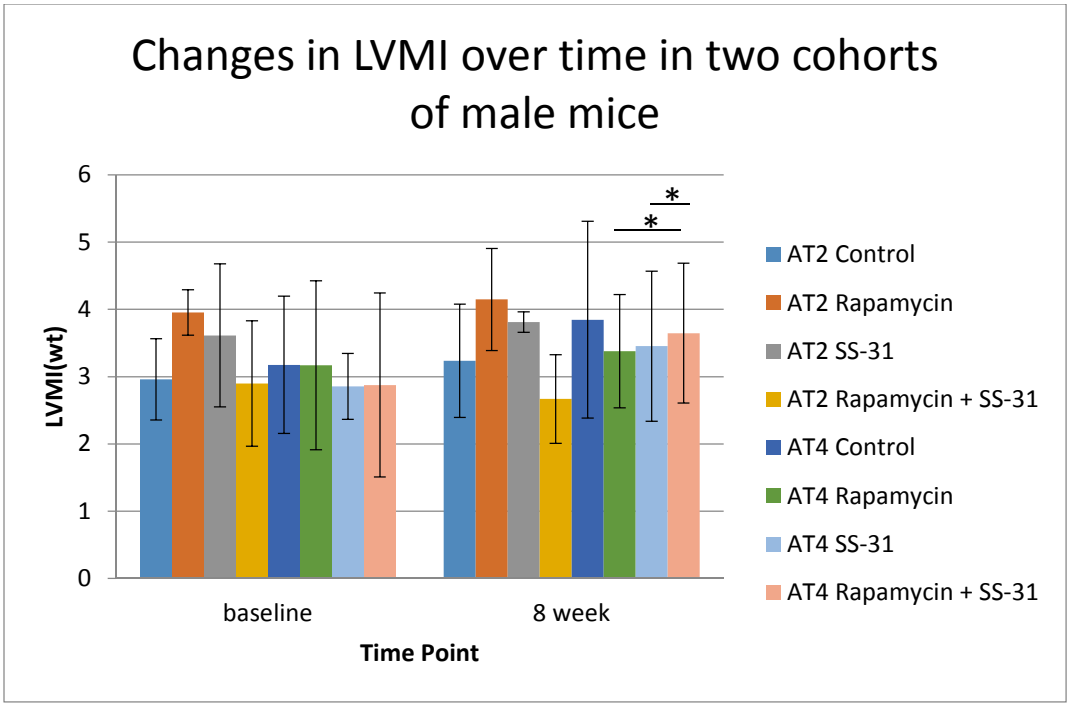


Figure 8: Changes in LVMI were measured for both cohorts of male mice. No significant changes were observed in the male mice in the first cohort. However, in the second cohort there was a significant increase in LVMI of the rapamycin + SS-31 compared to the rapamycin treatment and SS-31 treatment at eight weeks by unpaired t-test (*=p<0.05) (AT2 n=3-4; AT4 n=8-9). Error bars indicated standard deviation.

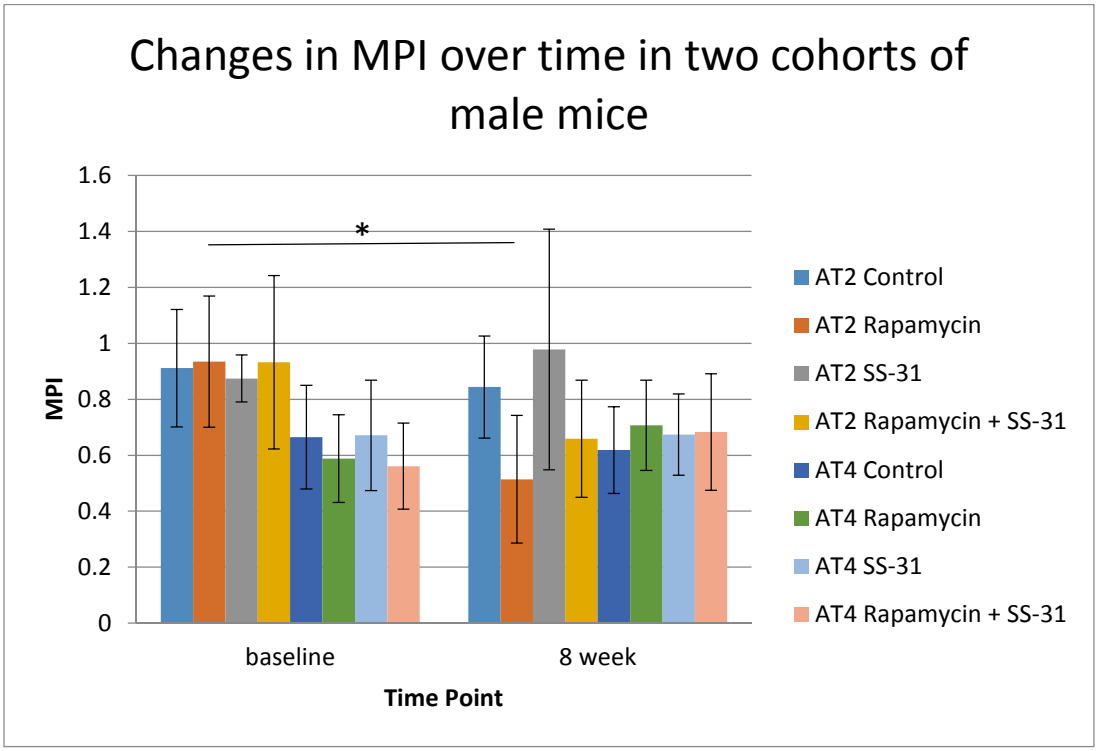


Figure 9: MPI was measured in two cohorts of male mice. There was a significant decrease in the MPI in the rapamycin treated mice after 8 weeks of treatment by paired t-test in the first cohort of male mice (*=p<0.05). There were no significant changes in the second cohort of male mice (AT2 n=3-4; AT4 n=8-9). Error bars indicated standard deviation.

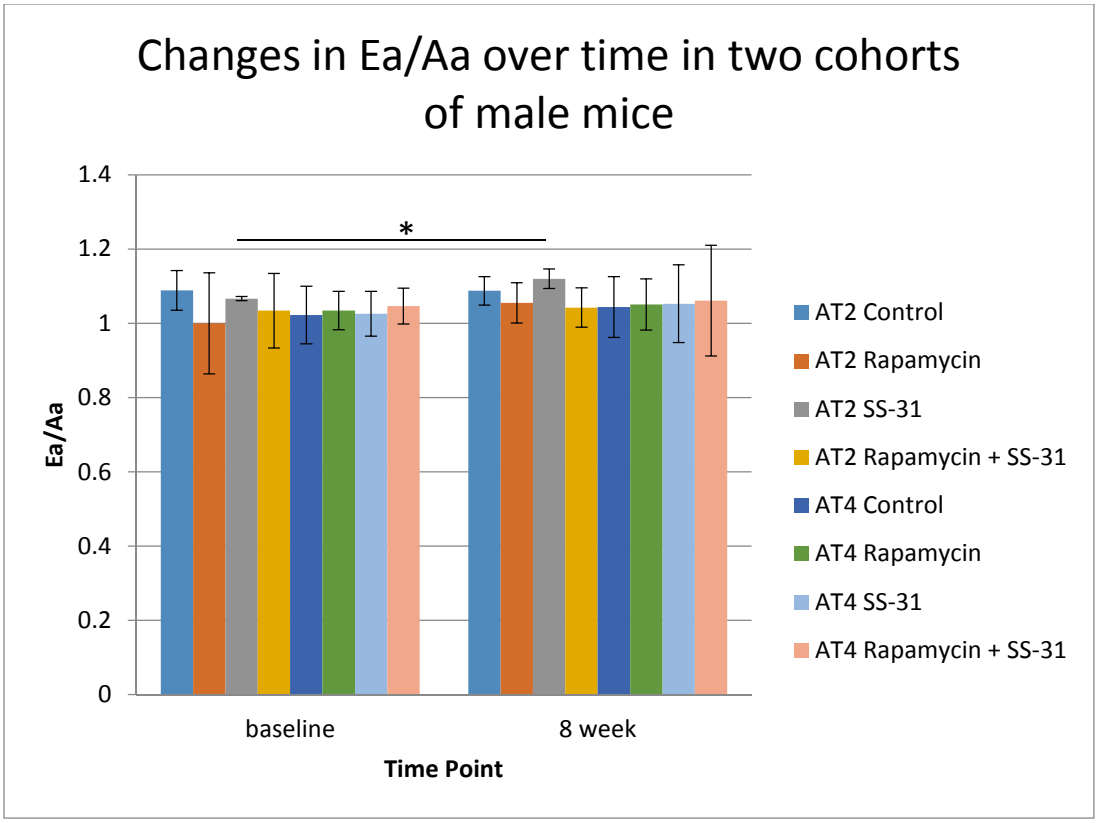


Figure 10: Changes in Ea/Aa measurements via echocardiography for two cohorts of male mice. In the first male cohort, there was a significant increase in Ea/Aa ratio in the SS-31 treated group after 8 weeks by paired t-test (*=$p<0.05$). No significant changes were seen between groups or over time in the second male cohort (AT2 n=3-4; AT4 n=8-9). Error bars indicated standard deviation.

In general, there are some interesting differences in the functional data that we observe between the three cohorts that were examined. The male cohorts had many significant changes in the fractional shortening and ejection faction, while the female mice had significant changes in the LVMI, MPI, and E'/A' measurements. In previous studies, we have not seen the changes that are observed in these male cohorts. In past work in the lab, ejection fraction and fractional shortening generally remain constant. The changes in the female cohorts are more typical. However the general patterns seen in these three cohorts between the genders might give an indication of changes that are gender specific. These changes could reflect that fact that in humans, females are more likely to experience HF/pEF compared to males, which is what the current mice exemplify.

The data from this experiment varies from previous experiments in some significant ways. Previously, SS-31 and rapamycin showed an improvement in both Ea/Aa ratio and MPI at both 4 weeks and 8 weeks compared to control. However, there are caveats to help understand some of the differences that we are observing. In the female cohort and the first male cohort (AT2) the number of animals was small, ranging from 3 to 4 animals per group, with one group that had 7 animals. These small numbers and biological variability have reduced statistical power to a level that solid conclusions are difficult. Additionally, echocardiography is an inexact science that takes many hours of practice before a true level of expertise is reached. The quality of echocardiograms improved over the course of time, but the initial study in the female mice was less than ideal. In the AT2 cohort, the author was experiencing some

health complications that could have adversely affected the quality of data obtained. The overall standard deviation was higher in all cohorts compared to previous studies. Finally, in comparison to previous studies, the mice in the male cohorts were blinded for data acquisition and data analysis in an attempt to remove any researcher bias. Taking all of these factors into account there are 13 comparisons between the three cohorts that approach significance ($0.05<p<0.1$), that might have led to a significant change, had a greater sample size and lower variability in measurement been obtained.

Heart hypertrophy measurements were taken at sacrifice time. Heart weight was normalized to body weight and tibia length in the female cohort of mice, but there was no significant change in either measurement, suggesting that there was no large change in heart hypertrophy between the various groups. In the two male cohorts, only heart weight normalized to body weight was collected. Similar to the female data, there was no significant change in hypertrophy in the first cohort of male mice. However, the large standard deviation in the SS-31 treated mice, might explain the lack of statistical difference. The second cohort of male mice, unlike the first male cohort or the female cohort had multiple significant changes. In this cohort, the rapamycin treated mice had significantly decreased hypertrophy measurements when compared to the control treated mice. Conversely, the SS-31 treated and rapamycin + SS-31 treated mice both had significant increases in their hypertrophy measurements compared to the control group. It is not clear why there might be an increase in the hypertrophy of the SS-31 and rapamycin treated mice, but it does suggest that SS-31 has a different and perhaps dominant effect on cardiac hypertrophy compared to rapamycin since the combined therapy mimics that of the SS-31 treated group and not the rapamycin treated group.

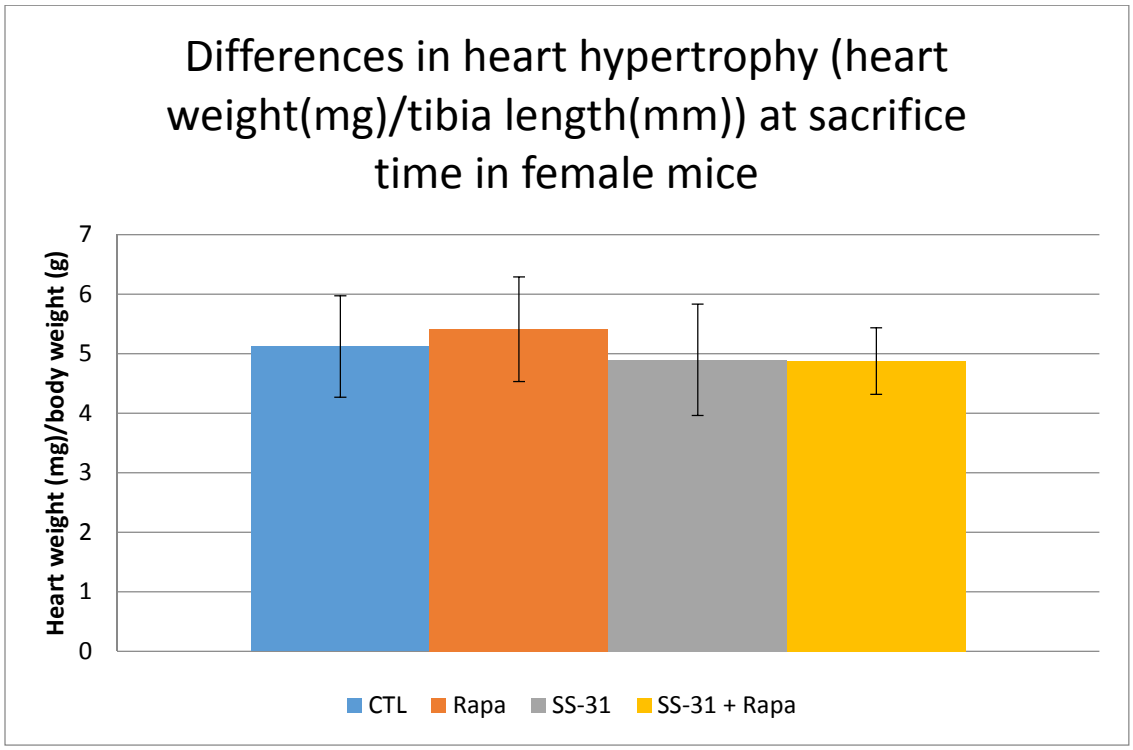


Figure 11: Measurement of hypertrophy from heart weight (mg) over body weight (g) at sacrifice time in the female cohort. No significant changes were found (n=3-7). Error bars indicated standard deviation.

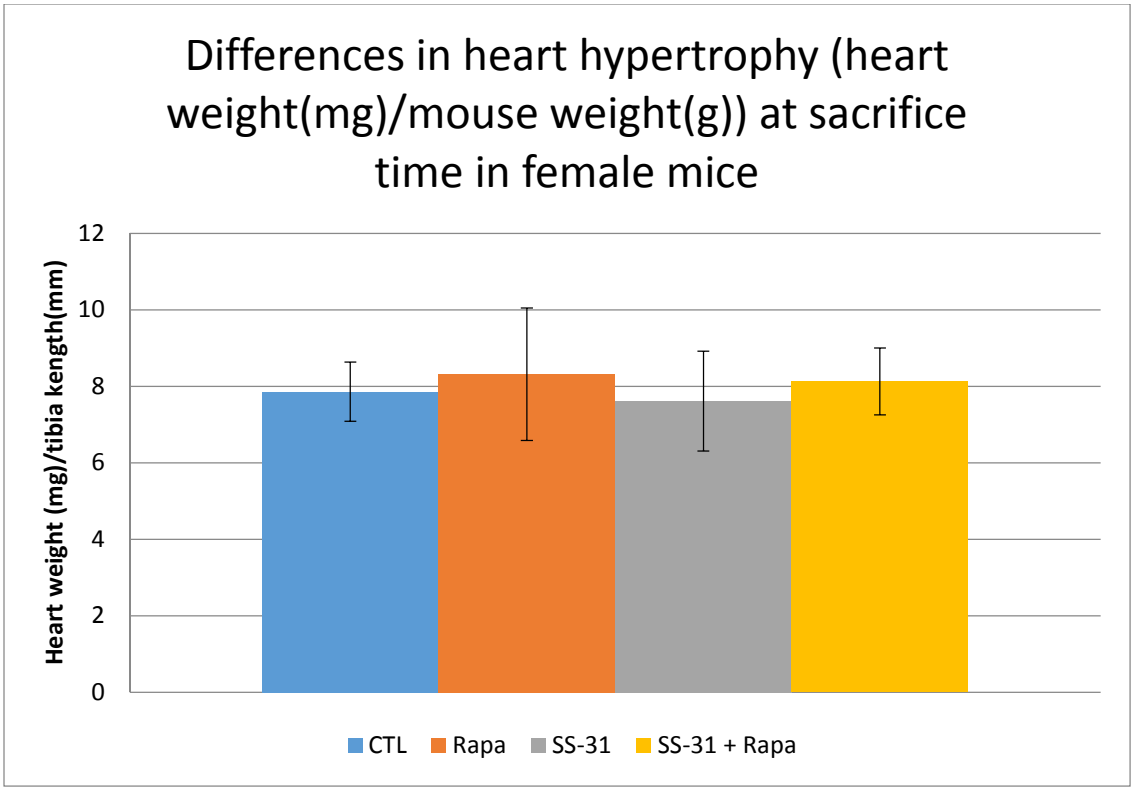


Figure 12: Measurement of hypertrophy from heart weight (mg) over tibia length (mm) at sacrifice time in the female cohort. No significant changes were found (n=3-7). Error bars indicated standard deviation.

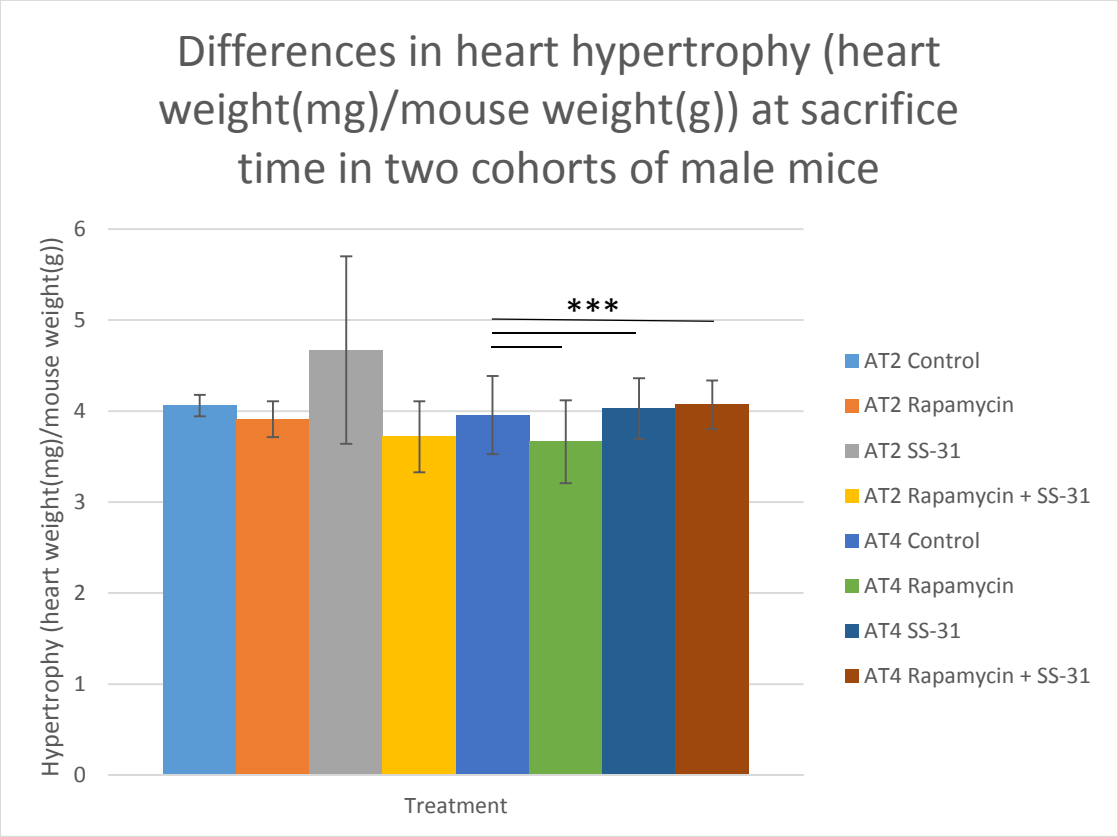


Figure 13: Measurement of hypertrophy from heart weight (mg) over body weight (g) at sacrifice time. In the initial cohort (AT2) there are no significant changes, however in the second cohort of mice (AT4) the changes in hypertrophy were significant in an unpaired t-test compared to the control group (***=p<0.001). Rapamycin treated hearts had a decrease in hypertrophy, while SS-31 and rapamycin with SS-31 increased in hypertrophy (AT2 n=3-4; AT4 n=8-9). Error bars indicated standard deviation.

II. In Vivo Studies (treadmill & grip strength)

Past work in Dr. David Marcinek's lab has shown that SS-31 treatment improves mice running times on a treadmill. Knowing this, we decided to see if the combined therapy might also (or further) improve the time to muscle fatigue in the mice. Mice were run on a mouse treadmill (Eco 3/6; Columbus Instruments, Columbus, OH, USA), after an acclimation evening, during their awake cycle at baseline and eight weeks. Running times were recorded and running distances calculated. As can be seen in figures 14 and 15 below, overall running time did not improve with any treatment of the mice for either the female or male cohorts. In our hands and with our instrument, motivation to run appeared to be a major variable in the assay. Endurance times were considerably lower than previously reported, and mice came to rest on, or partially on the shock plate, in spite of nudging according to protocol. Higher shocks strengths appeared to stun the mice, producing no greater endurance time.

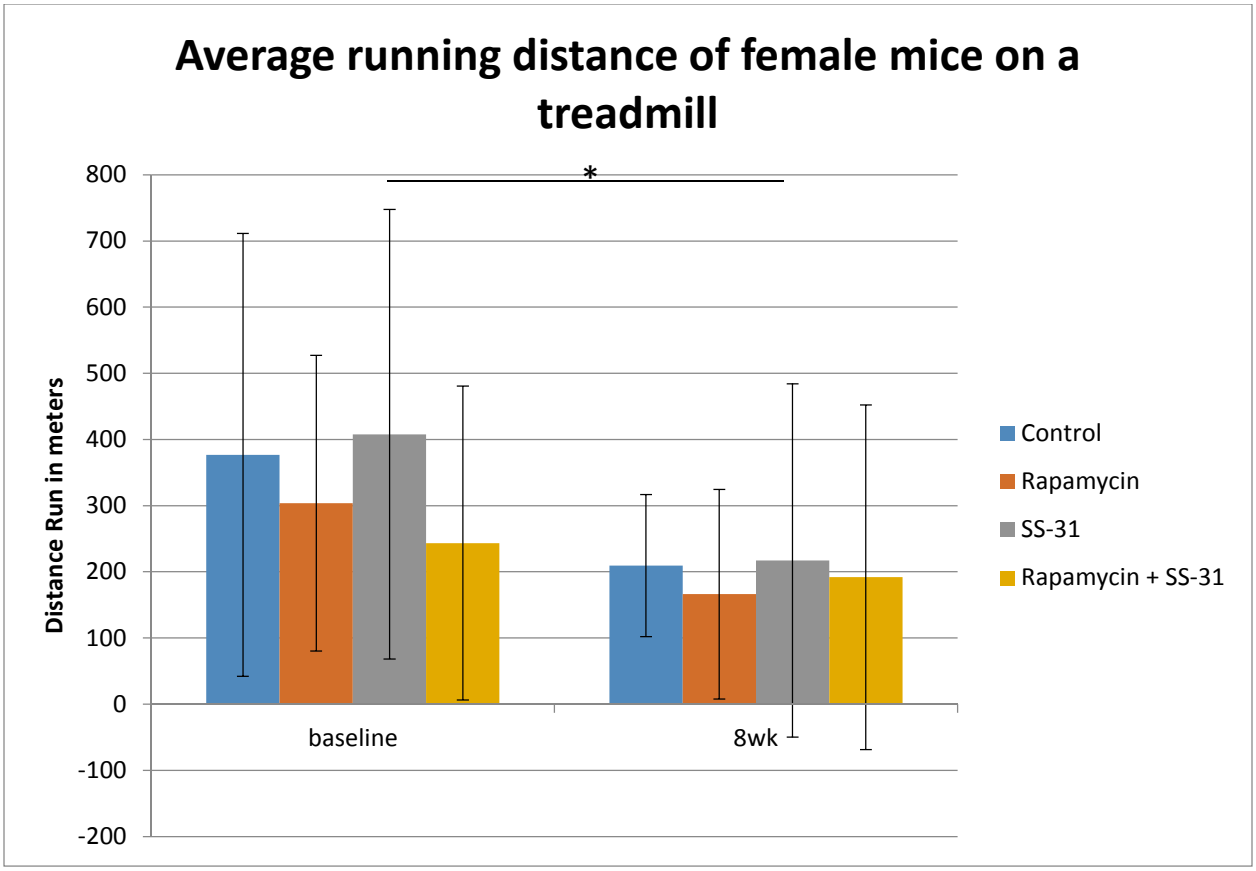


Figure 14: Average changes in mouse running distance over the treatment timeline in female mice. No significant changes were seen between the groups at baseline or at 8 weeks. There was a significant decrease in the distance mice ran at 8 weeks compared to baseline in the SS-31 treated group (*=$p<0.05$) (n=3-7). Error bars indicated standard deviation.

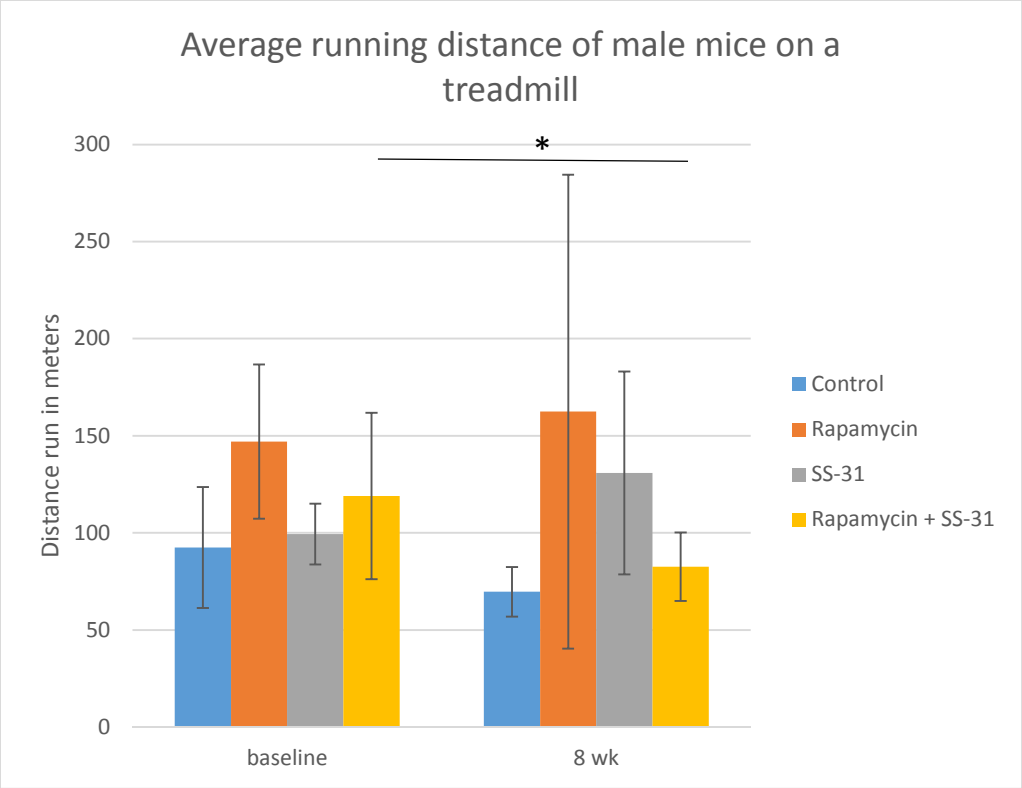


Figure 15: Average running distances on a mouse treadmill in a cohort of male mice. There was no significant change in running distance between treatment groups within time points. There was a significant decrease in running distance from baseline to 8 weeks in the rapamycin + SS-31 treatment group (*=$p<0.05$) (n=3-5). Error bars indicated standard deviation.

After seeing no change in running distance with the female mice, we decided to assess overall muscle strength using forelimb grip strength in the first cohort of male mice. Mice were given five trials to grip the GRIP STRENGTH apparatus with PC interface (San Diego Instruments, Inc.), with their highest strength recorded at baseline and eight weeks.

As can be seen in Figure 16, grip strength was lower at 26 months of age than at the baseline assessment 8 weeks earlier. While there were trends towards greater strength in treatment groups, variability in the data resulted in there being no significant change in grip strength for the mice regardless of treatment. Grip strength is difficult to measure consistently. The ability to grip the strength measuring bar and ensuring the mice get a strong grip every time is a difficult task. To help eliminate these inconsistencies, we gave mice five chances to grip the bar, and took the strongest of the five measurements.

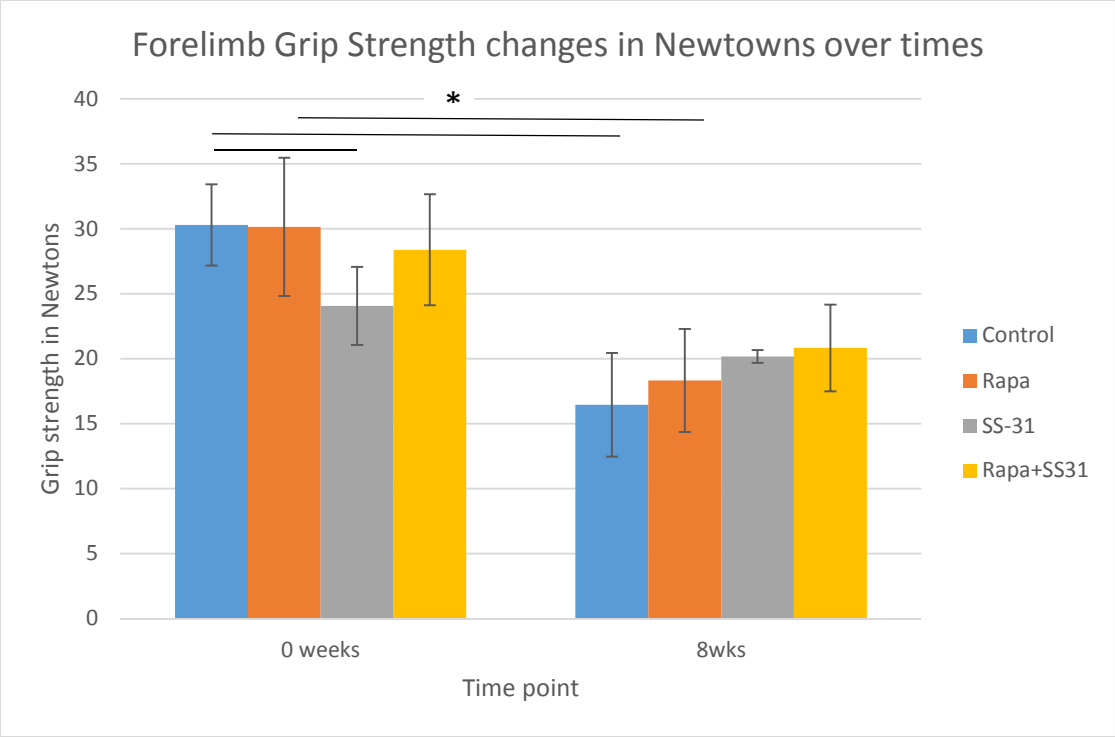


Figure 16: Mouse forelimb grip strength measurements at baseline and eight weeks. There is an initial difference between the control mice and SS-31 treated mice at base line that is significant in an unpaired t-test ($p<0.05$), while the only changes seen in eight weeks later are a significant decrease in strength in the control mice and rapamycin treated mice by paired t-test ($p<0.05$) ($n=3$-7). Error bars indicated standard deviation.

III. Ex Vivo Assessments

a. Proteomic changes by mass spectroscopy

After the sacrifice of the mice, heart tissue was powdered to prepare for multiple analyses. One of these analyses was proteomics by mass spectroscopy. To perform this, proteins were extracted from frozen heart tissues in ammonium acetate with rapigest, and then were trypsin digested and purified by MCX column (Waters). LC-MS/MS analysis was performed with a Waters nanoAcquity UPLC and a Thermo Scientific LTQ Orbitrap Velos, and the MS analysis of protein abundance was recorded. This was detailed in (Dai et al., 2014). Below we see some of the comparisons between the various groups.

Figure 17 shows the IPA pathway sorted z-score data for the 26 month old control, rapamycin, SS-31, and rapamycin + SS-31 for the female cohort (AT1, left hand side) and the first male cohort (AT2, right hand side). The red bars indicate that the z-score for the peptide was increased, while blue bars indicate a decrease. Additionally, the deeper the color, the stronger the increase or decrease. Proteomic analysis is an in-depth process that gives a vast amount of data, and can make it difficult to make simple conclusions. However, from looking at the figure, it is possible to not that one of the columns on each cohort is darker, and in the opposite direction of the others. In the female cohort, this is seen in the rapamycin treated group, while the male cohort shows the largest change in the rapamycin + SS-31 group. Within the AT1 cohort, the control group and the SS-31 treated group exhibit many of the same

changes in the same directions. While there are still changes in the overall z-scores of these proteins, the similarities between the two suggest that the role that SS-31 has on the proteome, are not as large as the rapamycin or the rapamycin + SS-31 treated groups. The rapamycin treated group mimics that of the rapamycin + SS-31 group, with changes seen in the same direction, but not to the same magnitude. In the male cohort, the proteomic changes are not nearly as clear cut as in the female group. While the control treated and SS-31 treated groups vary more than in the female cohort, the two groups are still the most similar to each other. In contrast to the female cohort of mice, the rapamycin + SS-31 treated mice had the largest change, and the changes observed were not similar to the rapamycin treated alone.

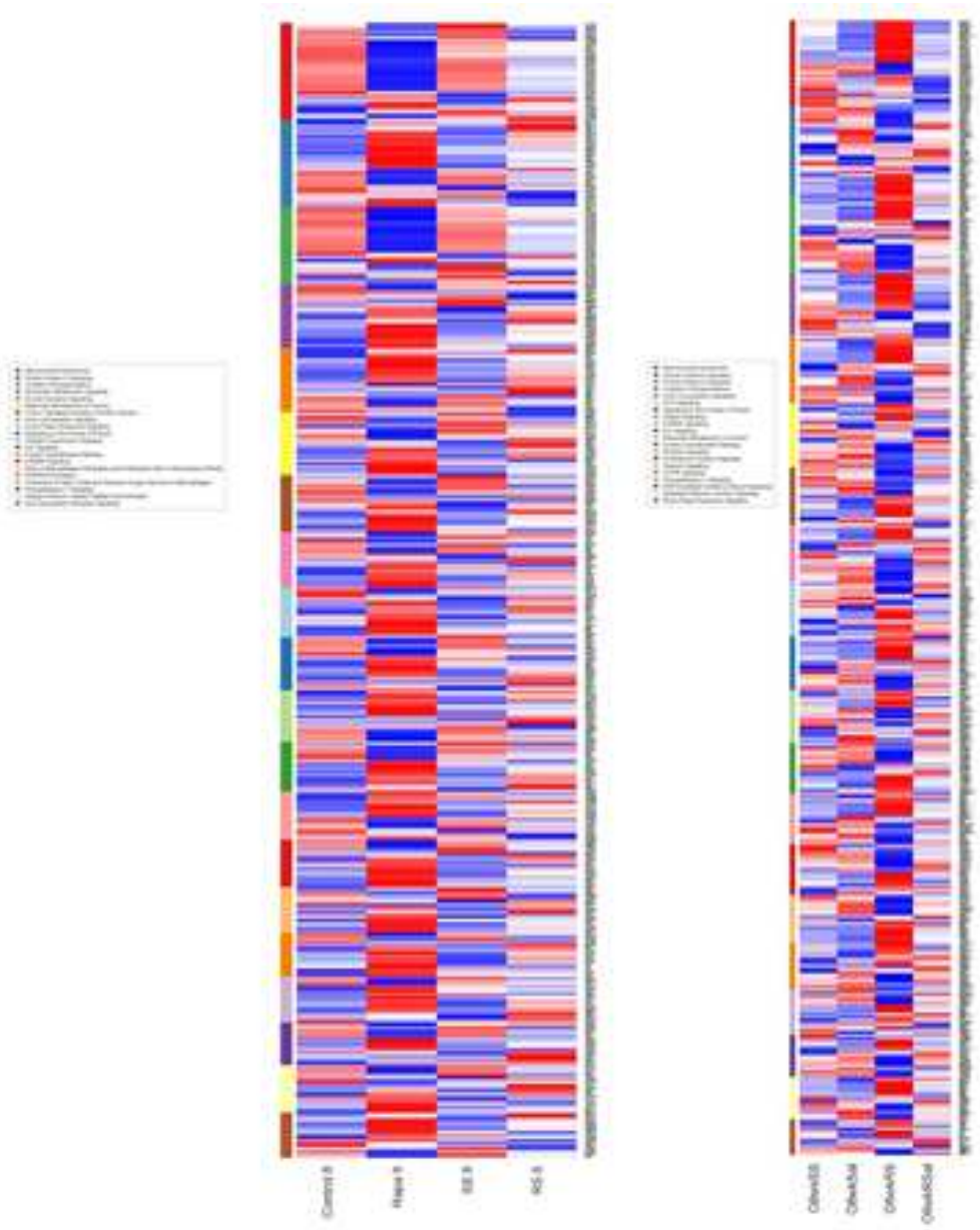


Figure17: Proteomic analysis from AT1 and AT2 by z-scores sorted into IPA pathways. The redder the panel, the more peptide was found in that treatment, the bluer the panel, the fewer peptides were found. In AT1, the largest

change was seen in the rapamycin treated group, while in AT2, the largest change was seen in the rapamycin + SS-31 group. Colored bars running along the left hand side indicate the IPA pathway the group of proteins is found it (n=3-7).

To gain insight into the proteomic changes, figure 18 isolates the first six pathways in the IPA pathway analysis. Again, the female cohort is on the left hand side of the figure, and the male cohort on the right side of the figure. Between the two groups, there is a difference in the pathways that are highlighted for change. Of the pathways, there are four that are the same between the two cohorts (mitochondrial dysfunction, protein kinase A signaling, axonal guidance signaling, and oxidative phosphorylation) and two that are unique. In the female mice, the unique pathways are xenobiotic metabolism signaling and molecular mechanisms of cancer. For the male cohort of mice, the unique pathways are actin cytoskeleton signaling and EIF2 signaling. While these pathways are unique in the first six pathways for IPA changes, this does not mean that these pathways are unchanged in the opposite gender, but rather that if they are changed, it is to a lower degree than in their counterpoint. Of the pathways that are the same, the relative z-scores for the proteins seem to go in the opposite direction. The best example of this is in the mitochondrial dysfunction pathway. In the female cohort of mice, the group that is most changed compared to the rest of the groups has a decrease in z-score compared to the other groups, while in the male cohort, the most changed group has an increase in z-score compared to the other three treatment groups that have a decrease in z-score. Overall, these images allow us to highlight the differences not only between the groups but also to see general trends between the genders.

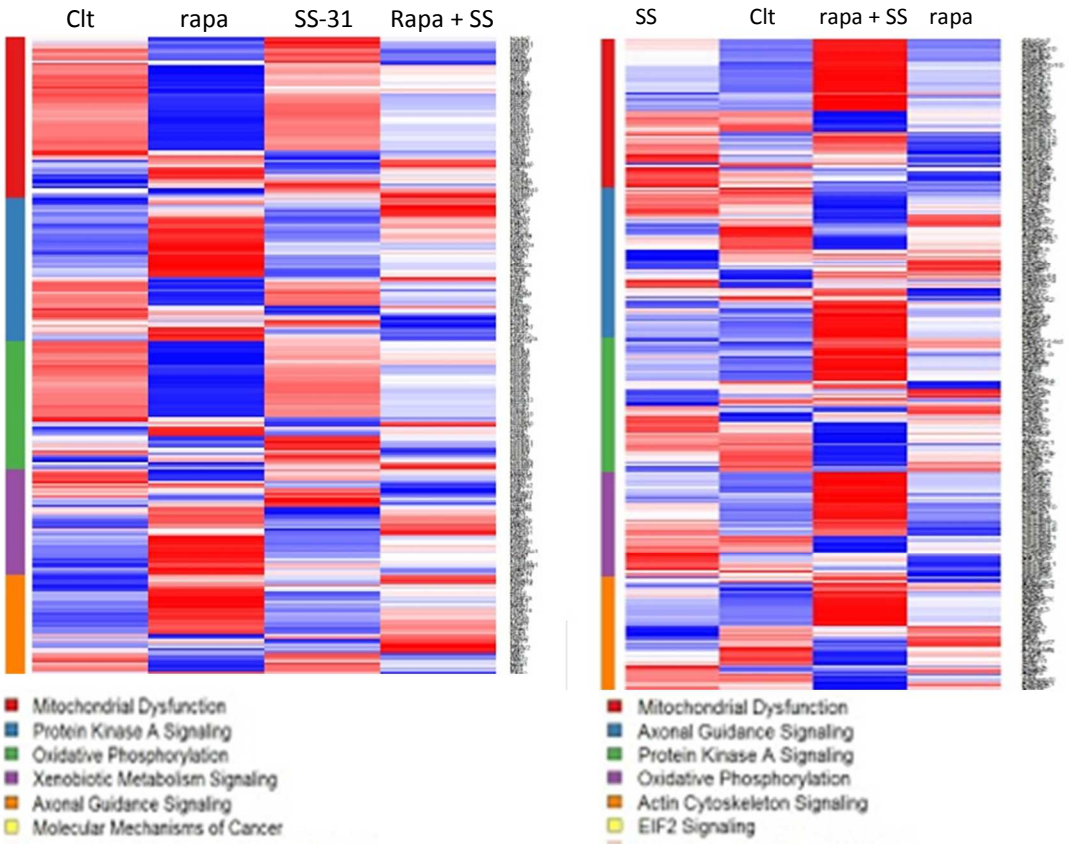


Figure 18: AT1 and AT2 magnification of the top part of the IPA pathway sorted images. The protein categories vary between the female and male treated groups, as can be seen by the colored groups and the respective legends for each group. Again, we see the rapamycin treated and rapamycin + SS-31 treated groups have the strongest z-scores, respectively (n=3-7).

While the data in figures 17 and 18 is valid, the process that separates the proteins into various pathways does not take into account the duplicating of proteins. For example, this means that many of the proteins we find in mitochondrial dysfunction pathway are the same proteins that we find in oxidative phosphorylation pathways. In figure 19, these duplications are eliminated, and the relative relationships and similarities to other pathways are shown. Knowing this, it is possible to confirm many of the earlier conclusions that were made about the relationships between the groups. In the female cohort of mice (left hand side of the figure), the control group and the SS-31 group share many of the same changes, and are more similar to each other than they are to the other two treatment groups. Additionally, the rapamycin treated group, has the furthest relationship away from any of the other groups. In contrast, the male mice show that the rapamycin and control group have the most similar proteomic profile, a fact that is skewed in the pathway data by the repeating proteins. However, the protein pathways did highlight the large change that is seen between the other groups and the rapamycin + SS-31 treated group. It should be noted, that being more closely related does not mean that there are no changes in the z-score. Despite the relationship stating that they are most similar, there are obvious differences between the most similar pathways. These changes highlight the relationship and general changes that we observe in every group, and allow us to look at general trend differences across studies, but still do not answer exactly how or what changes consistently.

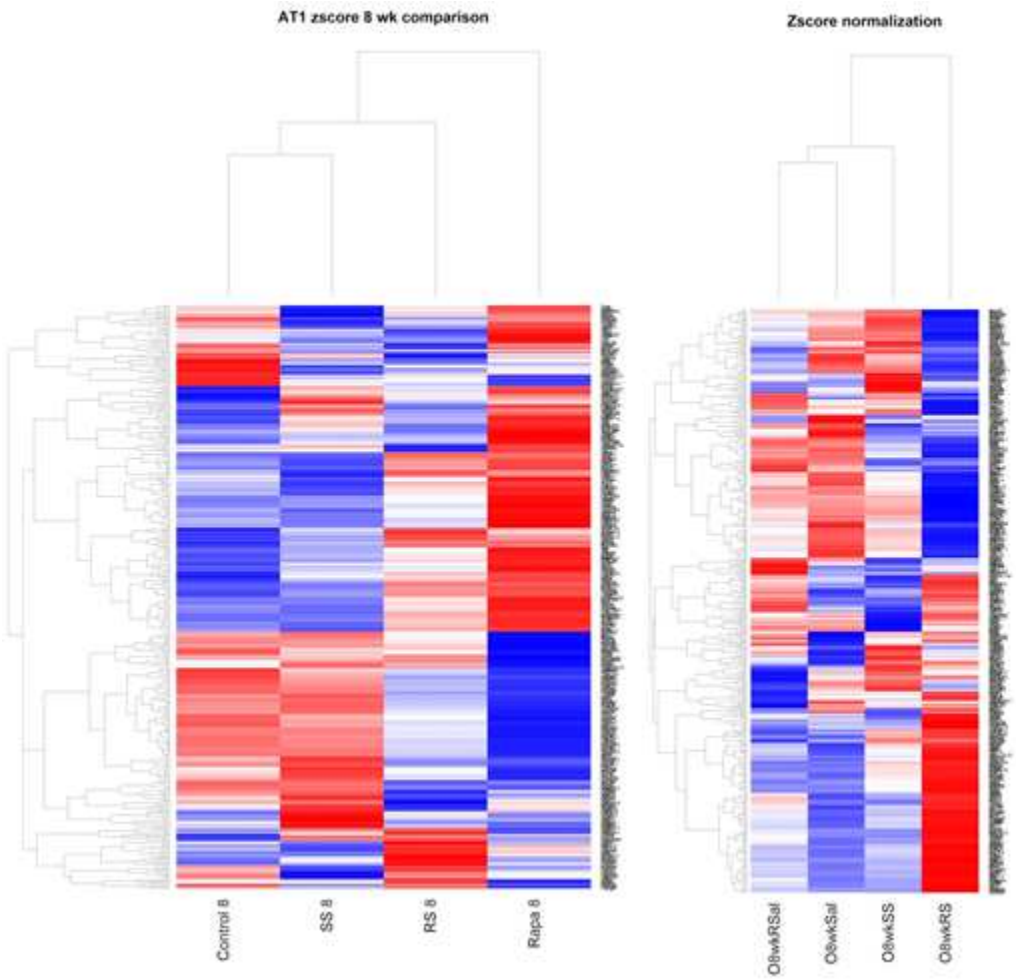


Figure 19: Mass spectroscopy proteomics data for a female (left) and male cohort of mice, based on relationship similarities and deviations. The more recently the lines diverged, either above for the treatment groups, or to the side, for the proteins, is a relation of how similar or dissimilar the groups are (n=3-7).

While proteomics is a powerful tool to investigate many protein changes as once, there are many complications that are still trying to be solved and minimized. Within the proteomic data gathering, there is loss of certain categories of proteins. One such group is insoluble proteins that are not included in this analysis, possibly leaving out relevant information on how the treatment affects the mice. The comparisons that are made across different cohorts, being measured during mass spectroscopy runs, cannot be directly compared, but rather can only help highlight general trends that occur between the groups and genders in different cohorts. Experimental differences occur with every data acquisition. The loss of proteins begins almost immediately after death, and the amount of time it takes to gather information during the time of sacrifice about the heart before it's placed in liquid nitrogen could affect the proteomics that are observed. While these changes are kept to a minimum, they cannot be completely ignored in the effect they might have on the proteomics. Finally, the number of mice in each cohort was low with AT1 having 3-7 mice per group, and AT2 containing 3-4 mice per group. While these numbers give a good start to the proteomic changes, with biological variability it is hard to see specific changes. Many of these challenges will be overcome in future studies, by an improved understanding of isolating all peptides, as well as increasing biological samples.

a. Protein levels by Western Blot

To investigate the protein levels, some of the powdered heart tissue was placed into lysis buffer where proteins were extracted. Those protein samples were then run on gels and stained with antibodies to look at protein levels. Five different antibodies were run on the blots, with each individual band being normalized to the whole protein stain for its sample, and then normalized to changes in protein level compared to young control.

The first blot that was run was a blot for oxidative phosphorylation. The antibody used was a cocktail of five different proteins, one from each mitochondrial complex. The five proteins are NADH dehydrogenase (ubiquinone) 1 Beta sub complex 8 (NDUFB8) found in complex I, succinate dehydrogenase subunit B (SDHB) found in complex II, cytochrome B-c1 complex subunit 2 (UQCRC2) found in complex III, cytochrome c oxidase subunit 2 (MTCO2) found in complex IV, and ATP synthase subunit alpha (ATP5A) found in complex V. While there are very few significant changes between the different treatments and the individual complexes, the small changes observed here help to understand some of the changes we see in the proteomics data, but still leave many avenues to explore for the full story.

In figure 20 the results from the NDUFB8 band are observed, normalized to the young saline control group. In this group, there is no significant change in any of the groups. However, there is a trend towards mice treated with either SS-31, rapamycin, or both, having a decreased amount of NDUFB8. NDUFB8 localizes to the intermembrane surface of complex I, and is thought to play a critical role in complex I due to its high level of domain conservation (Francis et al., 2014). The decreased amount of this protein in the treated group would suggest a decrease in the amount of complex I, the first step in oxidative phosphorylation. However, with the large standard deviation seen, and the standard error of means (not shown) suggest that the trends that are observed are not very strong and future work should be completed to get a better idea of the changes in NDUFB8 and complex I in general.

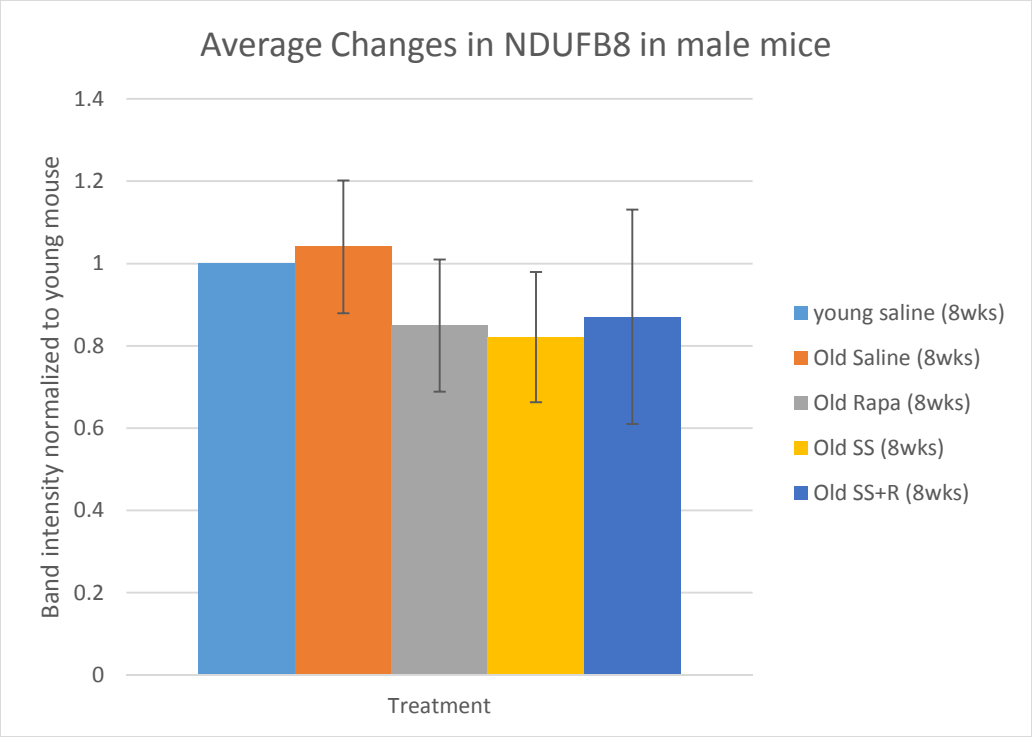


Figure 20: Average changes seen in male mice in oxidative phosphorylation protein NDUFB8 from complex I. There is no significant change between any groups (n=5). Error bars indicated standard deviation.

The second protein investigated comes from complex II, and the changes seen between groups can be observed in figure 21. Looking at figure 21, there are no significant changes, nor is there any general trends that can be observed. SDHB is an intermediate subunit, which is nuclear encoded, for complex II, interacting between the catalytic subunit A and the transmembrane subunits C and D (Saito et al., 2015). It appears that regardless of treatment, SDHB does not change drastically. This lack of protein level change suggests that complex II continues to function well as the mice age, and does not experience increased protein levels with therapeutic treatments.

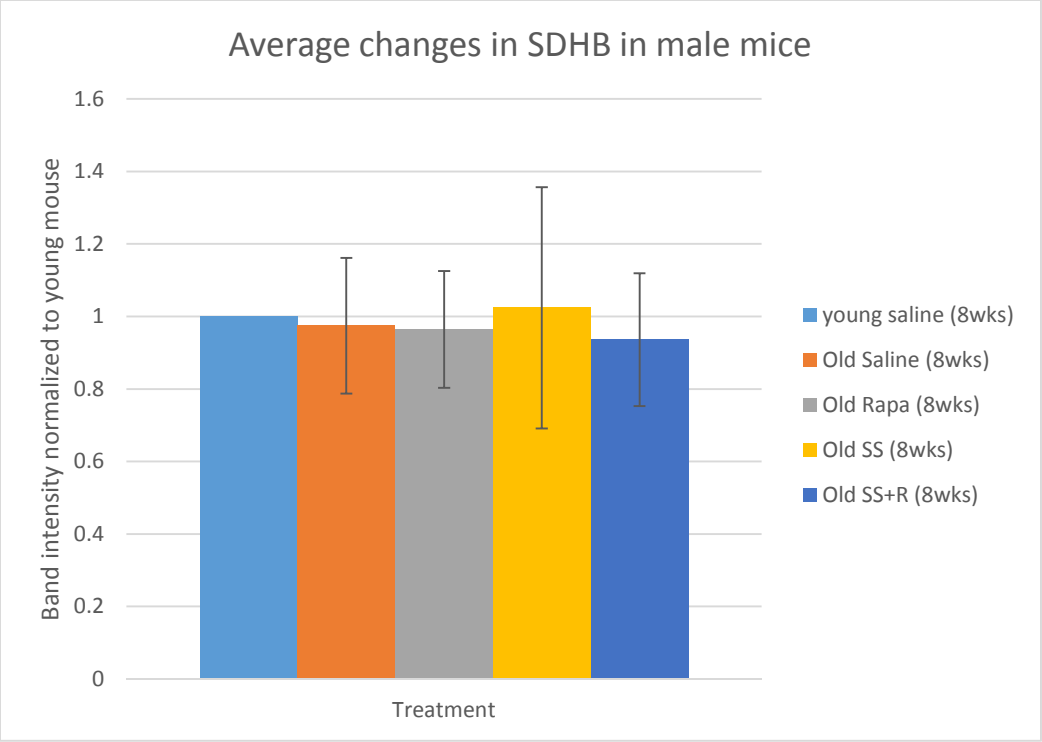


Figure 21: Average changes seen in male mice in oxidative phosphorylation protein SDHB from complex II. There is no significant change between any groups (n=5). Error bars indicated standard deviation.

UQCRC2 is a nuclear encoded protein in complex III of the electron transport chain (Miyake et al., 2013). When looking at figure 22, it is possible to see a significant decrease in the protein level in the rapamycin treated group compared to the young saline treated mouse. While there are no other significant changes, there is a trend towards a decrease in UQCRC2 in all the old male mice. Additionally, while rapamycin treated mice trend towards a decreased protein level compared to the old saline treated mice, the mice treated with SS-31 or rapamycin + SS-31 appear to have a higher level suggesting that SS-31 might help improve the amount of UQCRC2, improving the function of complex III compared to the old untreated mice.

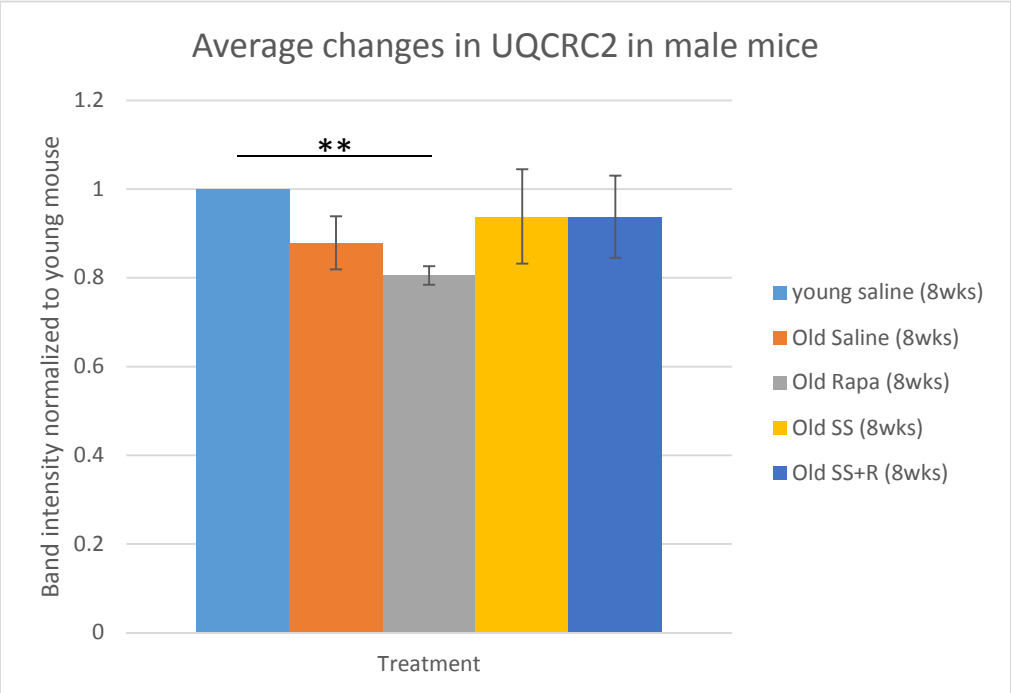


Figure 22: Average changes seen in male mice in oxidative phosphorylation protein UQCRC2 from complex III. There is a significant decrease in protein level in the rapamycin treated group compared to the young control (**=p<0.01) (n=5). Error bars indicated standard deviation.

MTCO2 plays a key role in transferring electrons from cytochrome c to oxygen in the catalytic subunit of complex IV (Szklarczyk et al., 2013). A decrease in the protein level suggests a decrease in the efficiency of electron transfer, causing electron leak and reactive oxygen species. In the male mice, there was no significant difference between any of the groups and the protein level of MTCO2. In more general terms, both treatments that contained rapamycin appear to have decreased the level of MTCO2 compared to the young control. This indicates that rapamycin might have an effect on complex IV of the oxidative phosphorylation chain. Additionally, the treatment with SS-31 only seems to have increased the level of MTCO2, perhaps giving an improved environment for the electron transfer. This result is in agreement with the published role of SS-31 interacting with cardiolipin to stabilize cytochrome c, thereby creating a more stable environment for the electron transfer. Interestingly, this beneficial aspect of the drug did not show any improvement when combined with rapamycin, suggesting that rapamycin interferes with this effect.

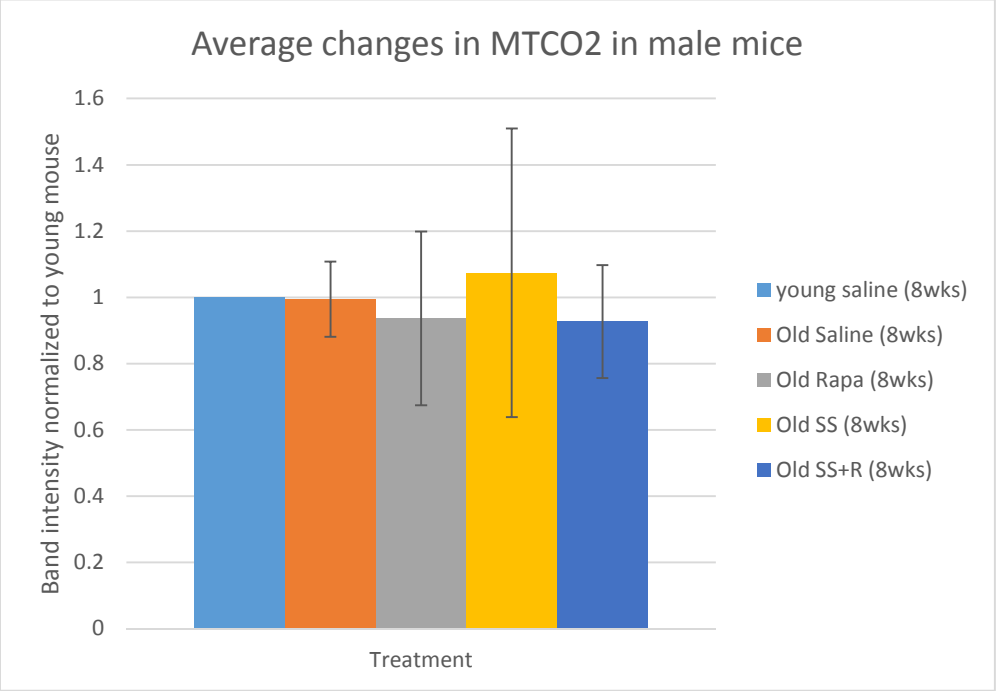


Figure 23: Average changes seen in male mice in oxidative phosphorylation protein MTCO2 or UQCRC2 from complex IV. There is no significant change between any groups (n=5). Error bars indicated standard deviation.

ATP5A helps convert ADP to ATP using the proton gradient in the final steps of oxidative phosphorylation (Wang et al., 2014). Without ATP5A there is an inability to convert ADP to ATP. Looking at graph 24, there are no significant changes in any of the groups. However, it appears that the old saline, rapamycin, and SS-31 treated groups trend towards an increase in ATP5A, suggesting that they might be able to complete more oxidative phosphorylation. Interestingly, the rapamycin + SS-31 group trended towards a decrease in ATP5A, suggesting that there is less oxidative phosphorylation occurring.

The trend towards an increase in the rapamycin or SS-31 alone treated mice, raises an interesting perspective when compared to the combined therapy that shows a decrease. This combined therapy might suggest that rapamycin and SS-31 interact in a fashion that decreases the ability of the oxidative phosphorylation chain to convert ADP to ATP.

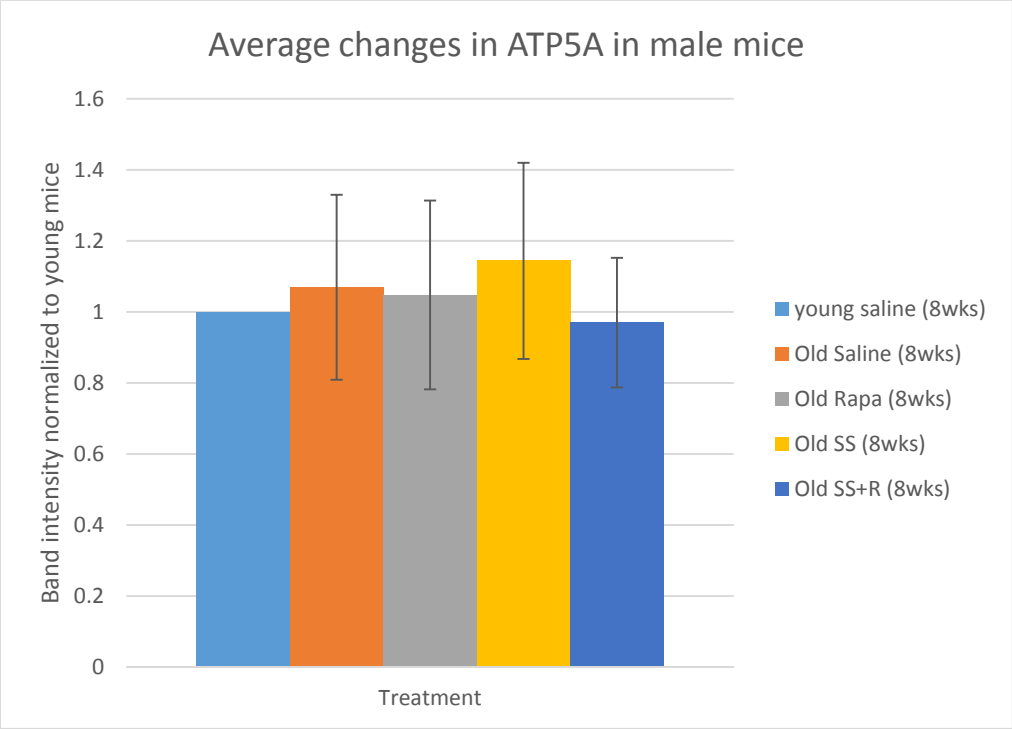


Figure 24: Average changes seen in male mice in oxidative phosphorylation protein ATP5A from complex V. There is no significant change between any groups (n=5). Error bars indicated standard deviation.

Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a protein that is often used as an inexact measure of autophagy (Tanida et al., 2008). The ratio of the two bands of LC3 (II and I) are an indication of autophagy. Using this protein for our studies, it is possible to have a general idea of the autophagy levels in the different groups as seen in figure 25. The only significant change seen is a decrease in LC3II/I in the SS-31 treated group compared to the young saline control. No other specific changes were seen, but there does appear to be a trend towards a decrease in LC3II/I in the older mice, regardless of treatment. This trend suggests that there is a decrease in autophagy, leaving more damaged cells and organelles in the heart, creating a less efficient environment.

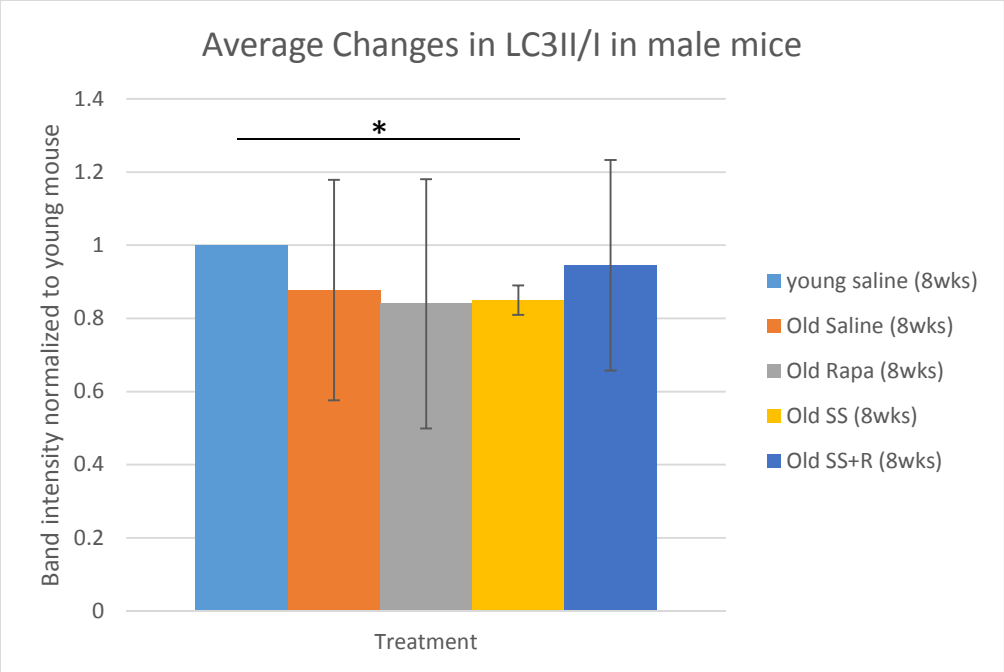


Figure 25: Average changes seen in LC3II/I levels of male mice. There is a significant decrease in the old SS-31 treated mice compared to the young saline treated mice (*=p<0.05) n=5). Error bars indicated standard deviation.

5'AMP-activated protein kinase (AMPK) is a protein involved in energy homeostasis and regulation (Mihaylova and Shaw, 2011). An increase in pAMPK compared to the total amount of AMPK is indicative of a switch to catabolic, or energy producing pathways (Mihaylova and Shaw, 2011). It also plays a role in cell growth and autophagy (Mihaylova and Shaw, 2011). In the current study, as shown in figure 26, there is a trend towards an increase in pAMPK/AMPK activity, suggesting an increase in catabolic pathways, autophagy, and cell growth. However, the only significant increase that is seen is in the rapamycin treated mice compared to the young saline mice. The pAMPK/AMPK data in all three of the old treated mice trending towards an increase suggests that, regardless of therapy, the two treatments helped the cardiac cells transition to a catabolic pathway, a more efficient energy producing pathway.

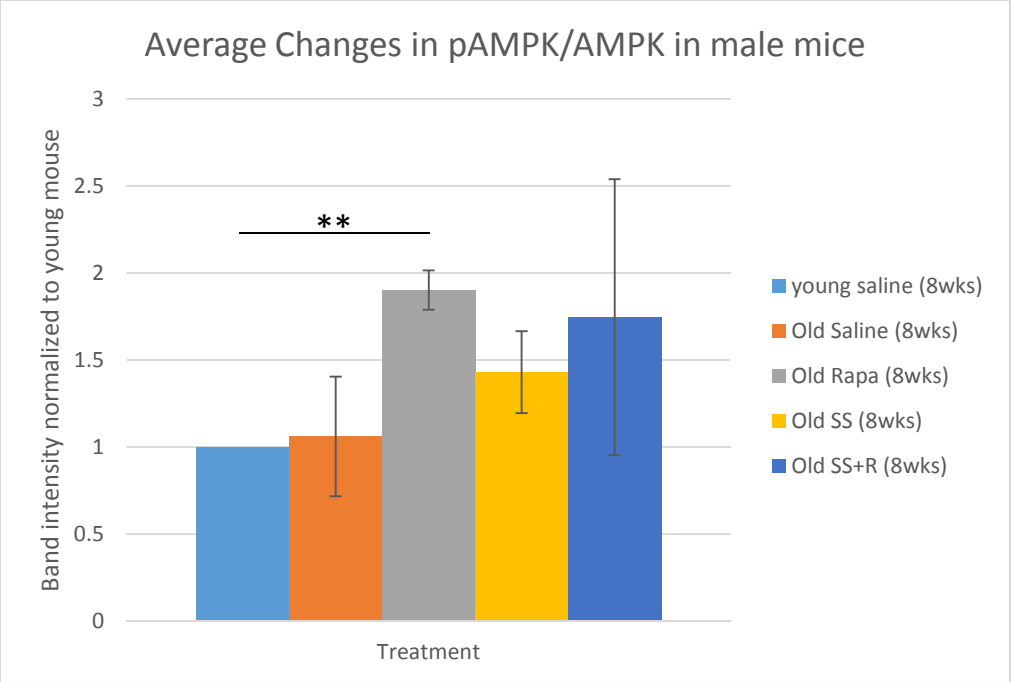


Figure 26: Average changes see in pAMPK/AMPK of male mice over various treatments. There is a significant increase in the rapamycin treated mice compared to the young saline treated mice (**=P<0.01) (n=5). Error bars indicated standard deviation. Error bars indicated standard deviation.

Peroxisome proliferator-activated receptor γ coactivator 1 (PGC1a) plays a central role in inducing mitochondrial biogenesis (Austin and St-Pierre, 2012). By looking the protein level, it is possible to deduce a general level of biogenesis. In the study of male mice, the cardiac tissue showed no significant changes as seen in figure 27. However, there might be a slight trend towards an increase in PGC1a in both the rapamycin treated group and in the SS-31 treated group. This trend suggests that with either therapy there is an increase in mitochondrial biogenesis in the heart. Considering this trend towards an improvement in PGC1a in each of the individually treated groups, it is surprising to see a possible trend towards a decrease in PGC1a in the combined therapy group. While this is very intriguing, more research needs to be done before a good understanding of what is occurring happens. Perhaps, the increase in the individual therapies feeds back to decrease the expression when both are given to the mouse.

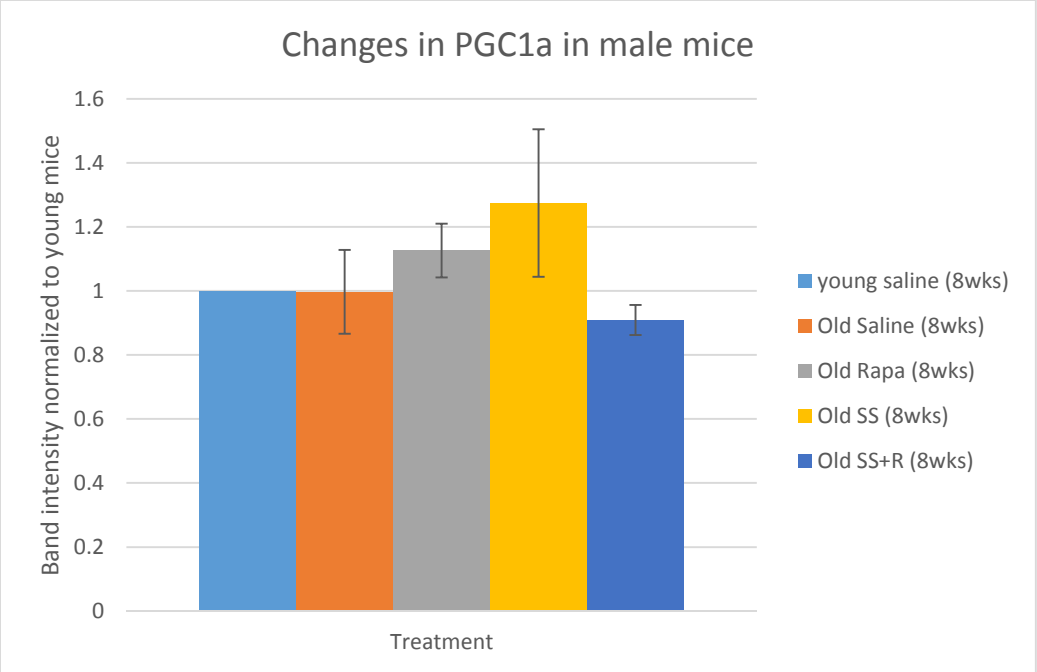


Figure 27: Average changes in PGC1a protein levels in male mice. There were no significant changes in any of the groups compared to each other (n=5). Error bars indicated standard deviation.

AKT is serine/threonine kinase that plays a key role in cell survival and proliferation (Song et al., 2005). A decrease in pAKT compared to AKT often indicates a decrease in proliferation and cell survival. Figure 28 the data gathered from the combined therapy treated mice is shown, there is a general trend towards a decrease in the pAKT/AKT protein levels. By way of significant changes, there is a decrease in pAKT/AKT protein level in the SS-31 treated mice compared to every other group minus the rapamycin + SS-31 treated group. Additionally, in the rapamycin + SS-31 treated group, there is a significant decrease in protein level compared to the young and old saline treated mice. This decrease suggests a decrease in proliferation of cardiac cells, which is expected both in heart disease and in older animals. The slight improvement in the rapamycin treated mice compared to the old saline controls is not completely unexpected since pAKT/AKT is in the mTOR pathway. The large decrease in the rapamycin + SS-31 treated mice, however, suggests that SS-31 has the more powerful effect compared to rapamycin in the combined treated group, and that the control of proliferation and apoptosis is not seen as strongly in this group.

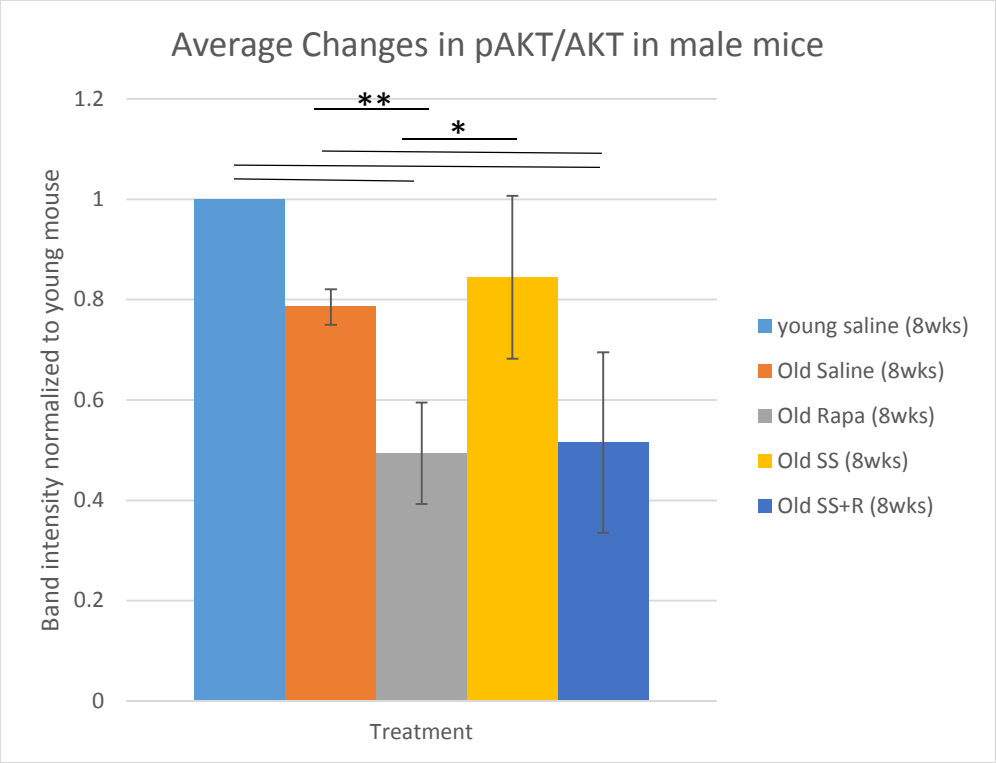


Figure 28: Average pAKT levels in male mice. There is a significant decrease in pAKT in rapamycin treated mice compared to young saline controls, old saline controls, and SS-31 treated mice. Additionally, there is a significant decrease in protein level of the rapamycin + SS-31 treated mice compared to the young saline treated mice and the old saline treated mice (*=$p<0.05$, **=$p<0.01$) (n=5). Error bars indicated standard deviation.

Overall, many of the protein levels change in opposite directions depending on the treatment and the role of that particular protein. The general meaning of these results is complicated by the large standard deviations and low number of animals. The western blots contained only five samples from male mice in each treatment group, which lacks statistical power to make a full conclusion. Future studies will have to increase this number. Additionally, the current studies have only been performed in male mice. As can be seen earlier in the proteomic and echocardiography data, it appears that male and female mice can vary in their reactions to different pharmaceutical treatments. Investigating the tissue and the role these proteins play in female mice will be essential.

IV. Conclusions

The combination of rapamycin and SS-31 gives results that were not expected. Initially, due to the divergent pathways that SS-31 and rapamycin act down, we expected to see an additive effect on the possible benefits that would be seen in the mice treated with both drugs. However, the functional data that we observed does not suggest a greater improvement in the combined therapy, and overall, the functional data that we collected does not recapitulate what we've seen in our studies in the past within the control groups (Chiao et al unpublished data). Additionally, we saw an increase in cardiac hypertrophy at the time of sacrifice in the combined treated mice and the SS-31 treated mice in the second cohort of male mice. The proteomic data has many interpretations. First, there is the difference

between the genders, with the female mice having a greater change in the rapamycin treated group compared to the treatment groups. The male mice, on the other hand, had the largest change in the rapamycin + SS-31 treated group, but the changes were in the opposite direction of the other groups. At this time, it is not possible to give a strong conclusion on the role of rapamycin + SS-31. Future work will continue to look at many of the changes already seen in the proteomics on a transcriptional level by qPCR and on the translational level using western blots. Additionally, earlier time points for many of the analysis that are seen will be necessary to investigate. In previous studies from our lab within the rapamycin groups, many of the greatest changes that were seen including biogenesis were seen at 1 and 2 week time points, and there were no changes at the final 10 week time point (Chiao et al unpublished data). A similar phenomenon could be seen in these studies and will need to be investigated in more depth. There is also the possibility that the combined treatment could show the greatest strength in stressed mice, such as probiotics having a greater effect on immune system improvement when mice are stressed (Palomar et al., 2014). The mice in this study were in a stress-free environment. Being contained in an SPSS facility decreased their stress to disease, and there was always amble food in the cages. The last possibility that could be investigated is that the improvement in many of the functions in the combined therapy group could have an adverse effect on other biological processes. Improving the health of the mitochondria to decrease cytochrome C oxidization, improving ATP production, and maintaining a healthier mitochondrial environment could inhibit other signaling pathways, similar to what is seen in young mice without any reactive oxygen species having inhibited immune system signaling (Basisty et al unpublished). Overall, the combination therapy looks to be having some additive effects on the treated mice, but more in-depth studies need to be performed, both in vivo and ex vivo.

III. SS-31 Persistence

I. Functional Assessments

Echocardiograms were performed in a similar manner to the combined SS-31 + rapamycin therapy study, but had additional time points added in order to assess whether the effects of SS-31 were persistent after withdrawal of the drug. Previously, our lab has shown that SS-31 treatment has improved MPI and E'/A' ratio at both 4 and 8 weeks post-treatment, suggesting that we might see an improvement at the later persistence time points as well. In this study, mice were echoed and fed control food as described previously in this thesis. Additionally, mice were echoed at 12, 14, and 16 weeks (with a 10 week time point in the female mice) to investigate if any functional changes were observed after SS-31 was removed at eight weeks.

In the female cohort of mice, there was no significant change in the ejection fraction or the fractional shortening, as can be seen in figure 29 and 30. There was a significant decrease in LVMI compared to baseline at 4, 8, 10, and 12 weeks in the control group, and a significant change in between the 8 week and 16 week SS-31 persistent groups as seen in figure 31. Most of the changes that occur in the MPI and E'/A' measurements are between groups, with a significant decrease in both as can be seen in figures 32 and 33. There are no significant changes between the two different conditions at any time points in MPI.

However, in the E'/A' measurements at 8, 10, and 14 weeks the SS-31 treated mice were significantly higher than the control mice.

These changes seen in the female cohort of mice suggest that many of the changes that are seen are aging changes within the same groups, and that SS-31 does not provide a persistent benefit in the mice nor did it have any effect at earlier time points. However, the in the E'/A' ratio, there is a suggestion that the SS-31 treatment improves E'/A' ratios while the drug is being administered, and possibly up to four weeks after the drug is removed. Future studies increasing the number of mice and decreasing the variability in measurements for the mice would highlight the exact persistent effect.

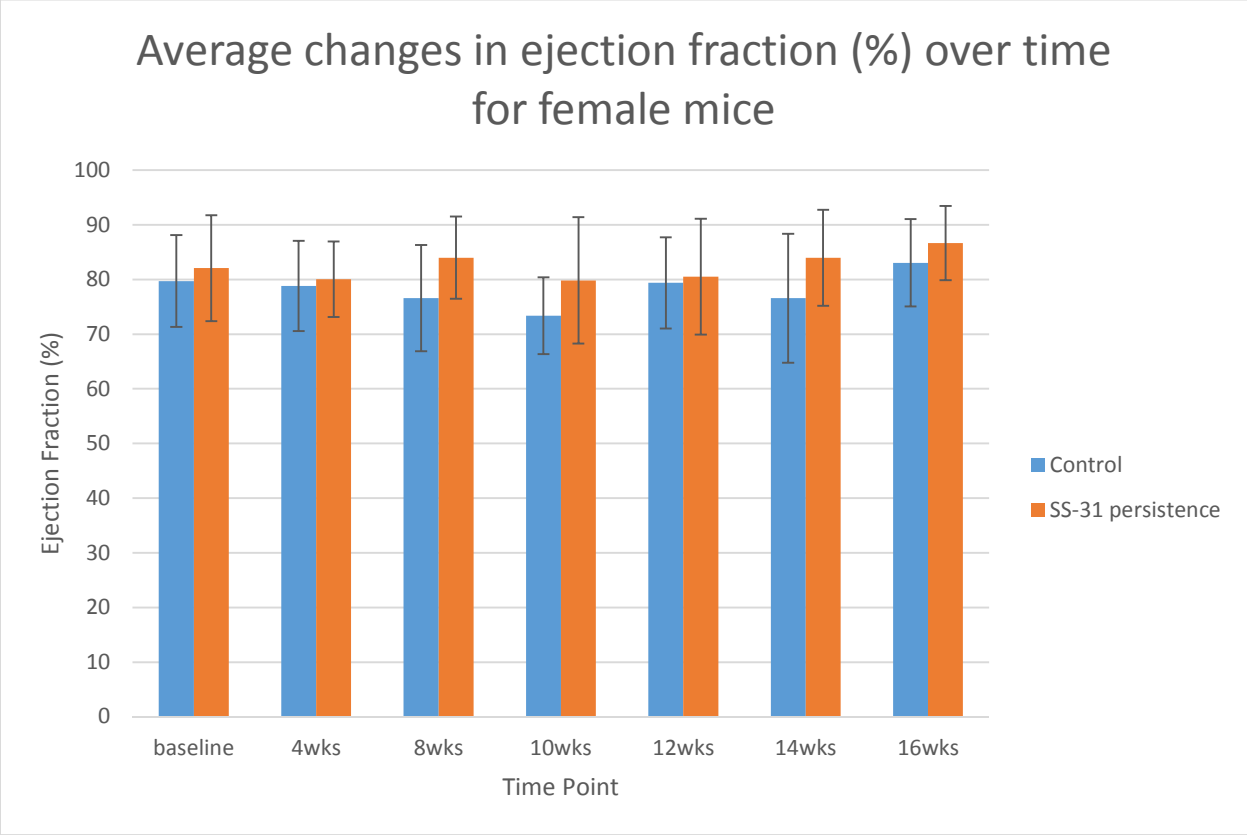


Figure 29: No significant changes were seen in the female cohort of mice in the ejection fraction measurements (n=6-9). Error bars indicated standard deviation.

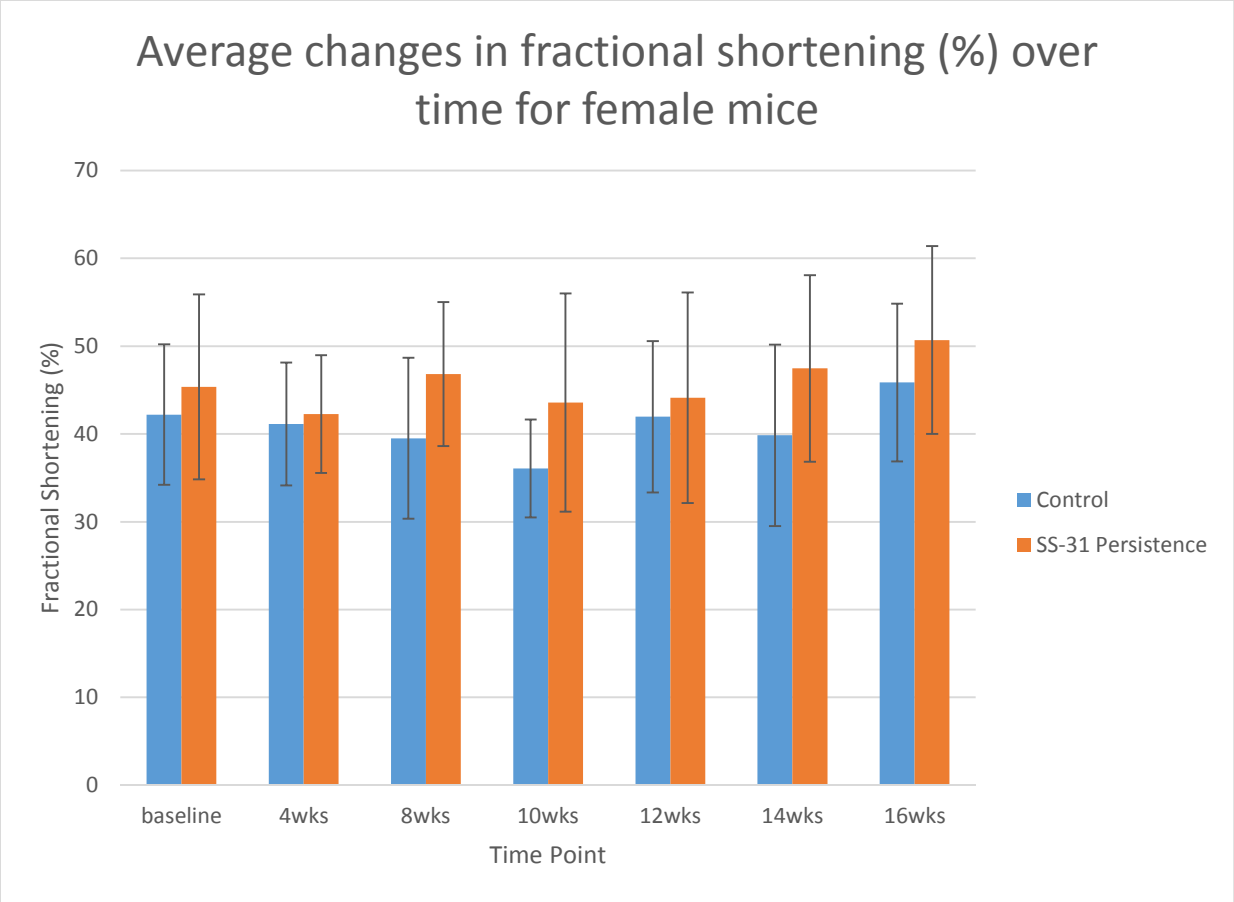


Figure 30: No significant changes were seen in the fractional shortening of the female cohort of mice (n=6-9). Error bars indicated standard deviation.

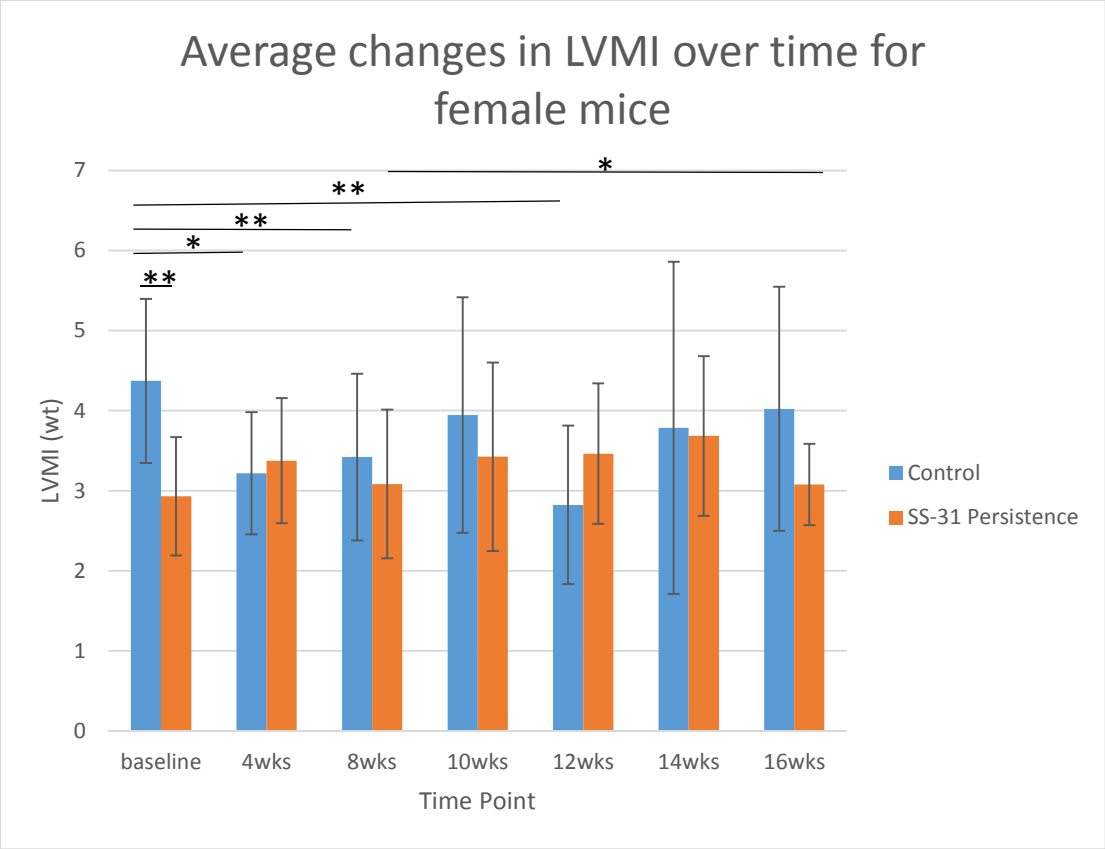


Figure 31: Changes in LVMI measurements via echocardiography for the female cohort of mice. There was a significant decrease by paired t-test between the baseline control measurement and the 4 week, 8 week, and 12 week time point (*=p<0.05, **=p<0.01). In the SS-31 treated mice, there was a significant change by paired t-test between the 8 week and 16 week measurement (*=p<0.05) (n=6-9). Error bars indicated standard deviation.

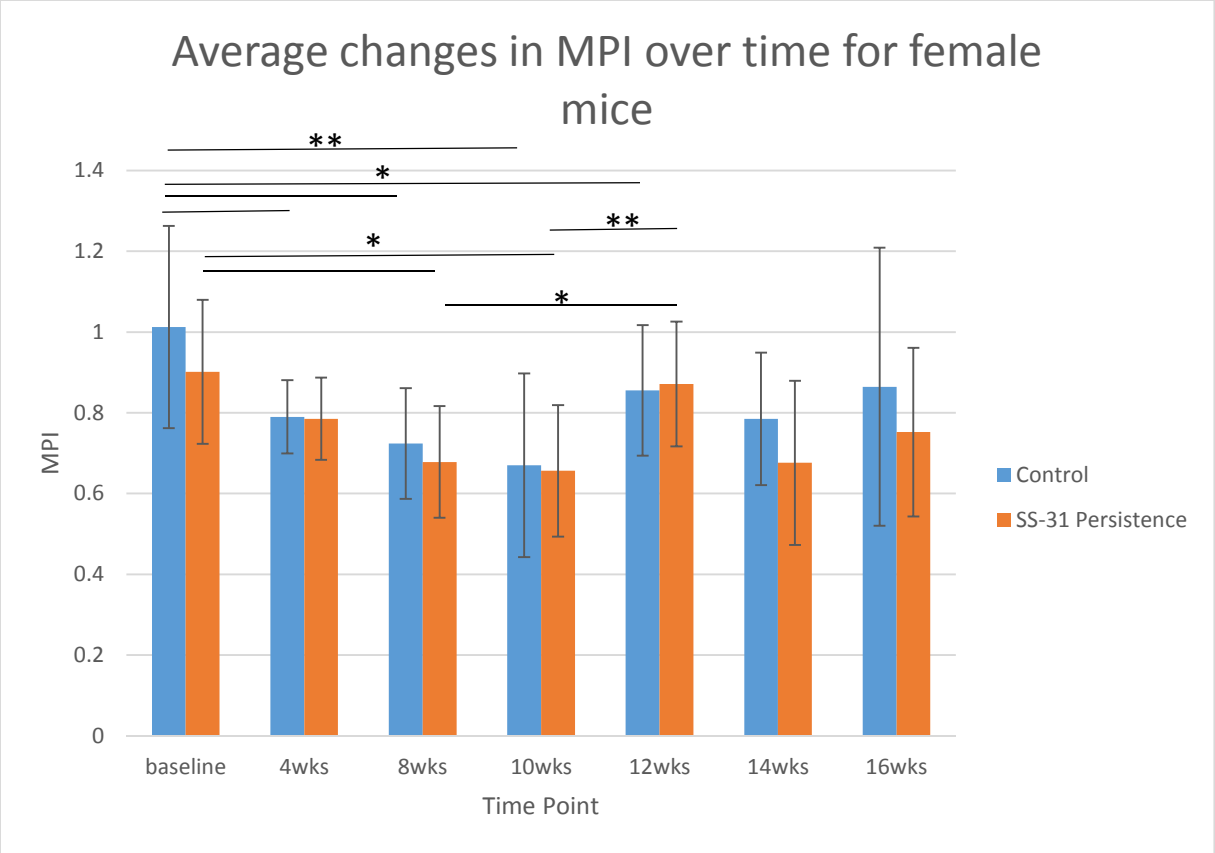


Figure 32: Changes in MPI measurements via echocardiography in female mice. In the control cohort, there was a significant decrease in MPI at 4 weeks, 8 weeks, and 12 weeks compared to baseline by paired t-test (*=p<0.05, **=p<0.01). In the rapamycin cohort there are some similar changes observed. MPI is decreased at 8 weeks and 10 weeks post-treatment compared to the baseline by paired t-test (*=p<0.05). Additionally, by paired t-test there is an increase in hypertrophy at 12 weeks compared to either 8 weeks or 10 weeks when treated with SS-31 (*=p<0.05, **=p<0.01) (n=6-9). Error bars indicated standard deviation.

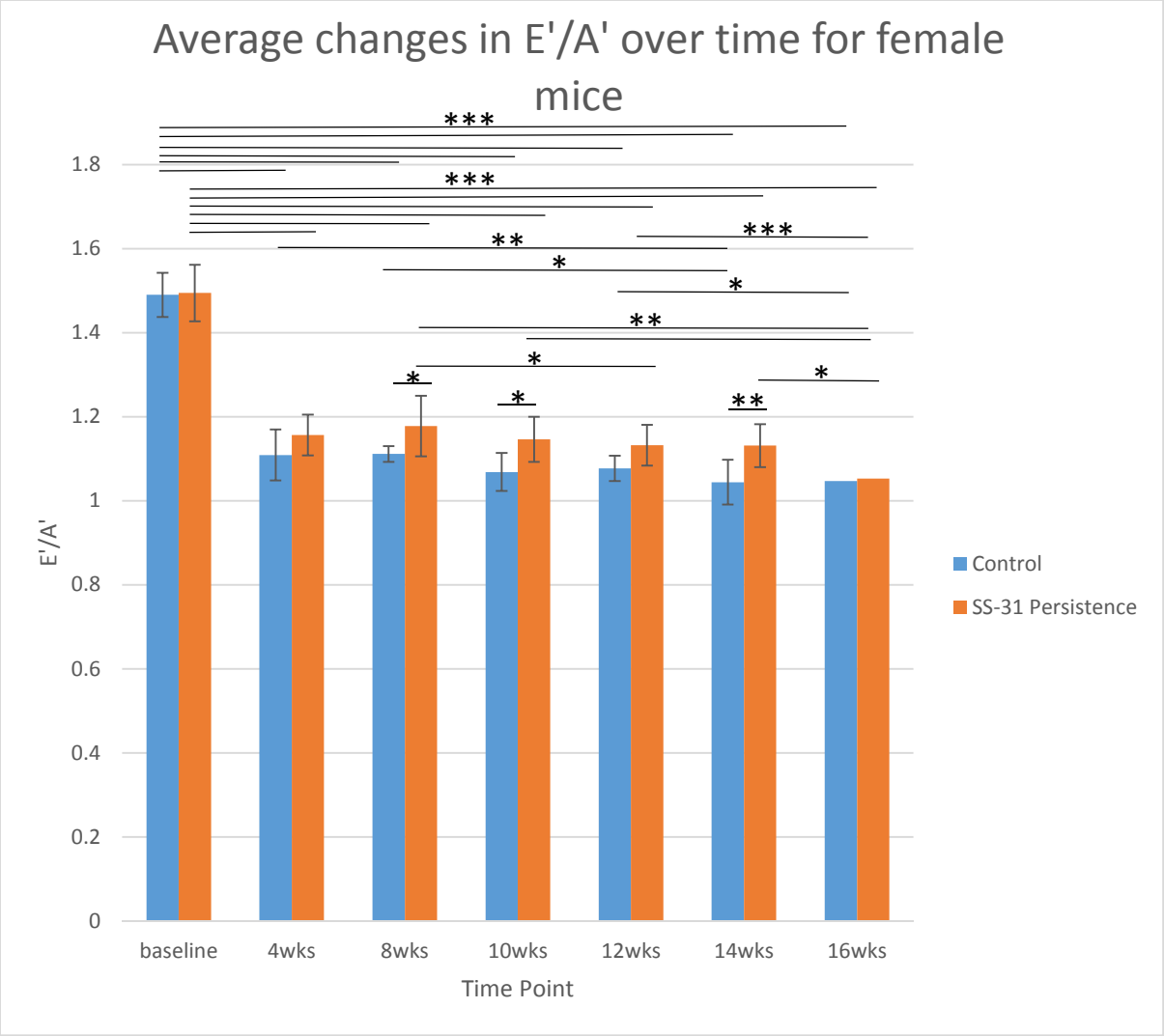


Figure 33: Changes in Ea/Aa measurements in female mice via echocardiography. In both the control and SS-31 treated mice, there was a significant decrease in E'/A' ratio at all time points compared to the baseline (***=p<0.001). In the control group, there was a significant decrease in E'/A' ratio at 14 weeks compared to 4 weeks, at 14 weeks compared to 8 weeks, at 16 weeks compared to 12 weeks by paired t-test (*=p<0.05, **=p<0.01). In the SS-31 persistence group there is a significant decrease at 12 weeks compared 8 weeks, 16 weeks compared to 8 weeks, 10 weeks, 12 weeks, and 14 weeks by paired t-test (*=p<0.05, **=p<0.01, ***=p<0.001). Finally, there is a significant increase in E'/A' in the SS-31 treated groups at 8 weeks, 10 weeks, and 12 weeks compared to the control treated mice at the same time points by unpaired t-test (*=p<0.05, **=p<0.01) (n=6-9). Error bars indicated standard deviation.

In the male mice, we see some significant changes in all the measurements. In figures 34 and 35 in the initial cohort (AT2) there is a significant increase in fractional shortening and ejection fraction in the control group from baseline to 16 weeks, and a significant increase in the SS-31 treated mice compared to the control mice at 8 weeks. In the second cohort, there was a significant increase in fractional shortening and ejection fraction at 16 weeks compared to 12 weeks. In LVMI measurements, the only significant changes were seen in the AT2 control group, with an increase at 8 week and 16 weeks compared to the baseline measurement, as can be seen in figure 36. The MPI is not significantly changed in the AT4 cohort, but in the AT2 cohort, there is a significant decrease at 8 weeks and 16

weeks compared to baseline in the control group, and at 8 weeks compared to baseline in the SS-31 treatment group as can be seen figure 37. Finally, as can be seen in figure 38, in the E'/A' ratios, there were no changes in the AT4 cohort, but there was a significant decrease at 8 weeks and 16 weeks compared to the baseline measurement for the control group for AT2.

Many of the changes seen in the male cohort of mice mimic that of aging, and don't generally reflect on the persistence of SS-31. MPI, E'/A', and LVMI (wt) maintain their baseline levels for almost the entire study periods. This suggests that SS-31 does not improve or maintain function of these aspects of cardiac aging in the male cohort. However, it does appear that there is a slight, but not significant increase in the measurements for ejection fraction and fractional shortening, which are similar to the results seen in the eight week cohorts, suggesting that SS-31 persistence might interact on these functions in the male mice.

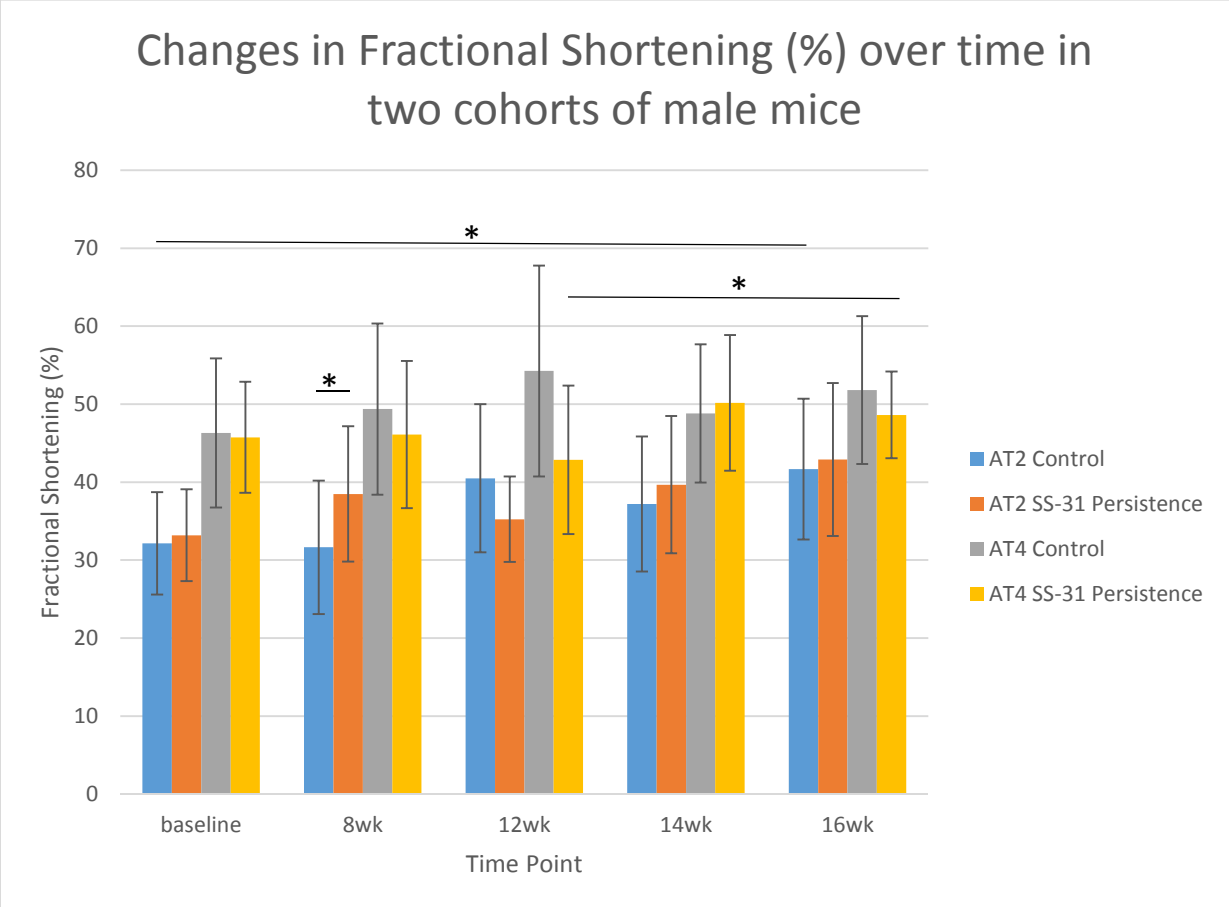


Figure 34: Changes in fractional shortening in two cohorts of male mice. In the initial cohort of male mice (AT2), there was a significant increase in fractional shortening from the baseline to 16 weeks in the control group by paired t-test (*=p<0.05). Additionally, within the first cohort of male mice, there is a significant increase between the 8 week control and 8 week SS-31 by unpaired t-test (*=p<0.05). In the second cohort, the only significant change that is seen is a significant increase from the 12wk SS-31 treated to the 16wk SS-31 treated by paired t-test (*=p<0.05) (AT2 n=10-11 ; AT4 n=10-12). Error bars indicated standard deviation.

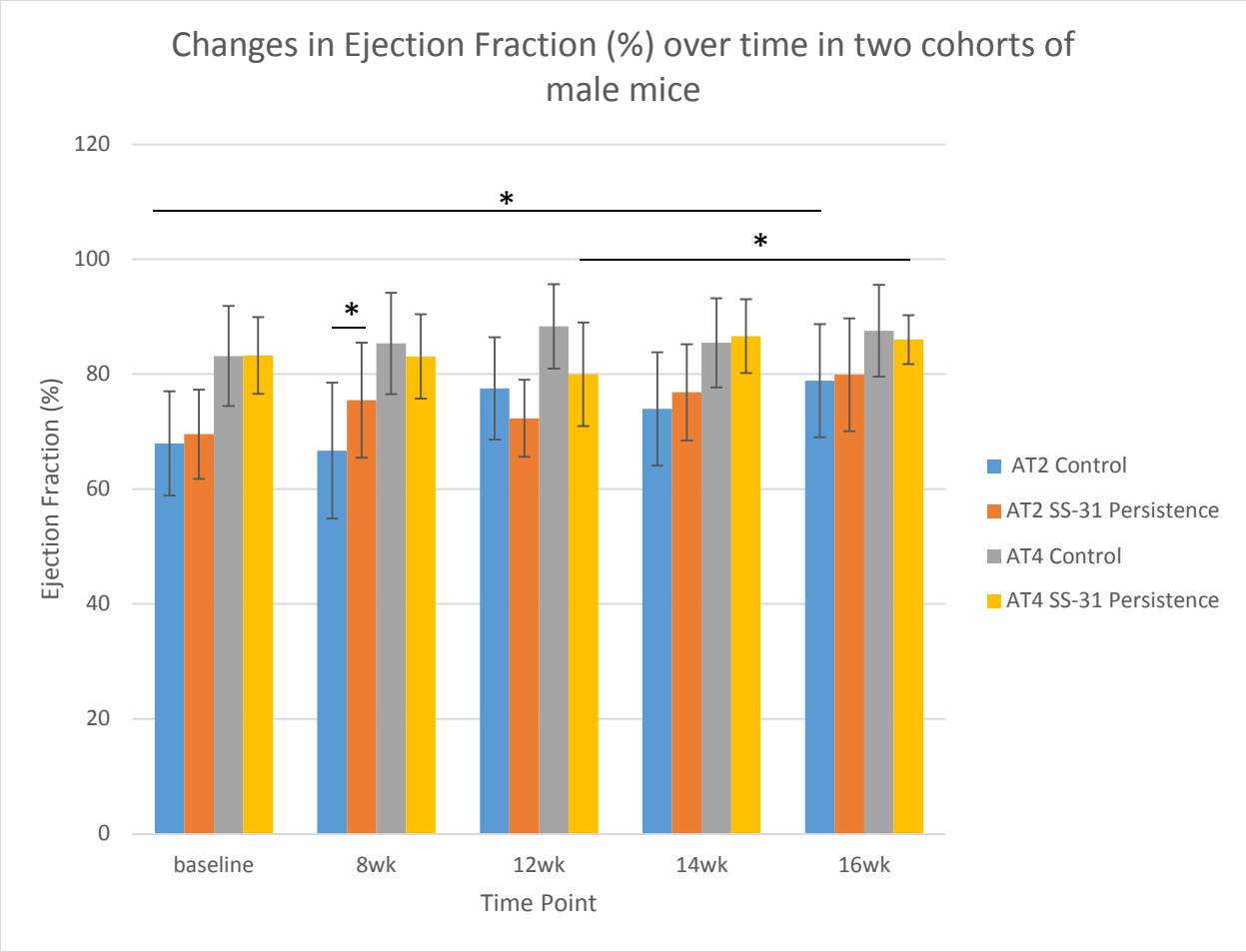


Figure 35: Ejection fraction changes in two cohorts of male mice. In the first cohort of male mice, there is a significant improvement from baseline to 16 weeks in the control mice by paired t-test (*=p<0.05). Additionally, there is a significant increase at 8 weeks in the rapamycin treated compared to the control by unpaired t-test (*=p<0.05). In the second cohort, there was an increase from 12 weeks to 16 weeks in the SS-31 persistence group by paired t-test (*=p<0.05) (AT2 n=10-11; AT4 n=10-12). Error bars indicated standard deviation.

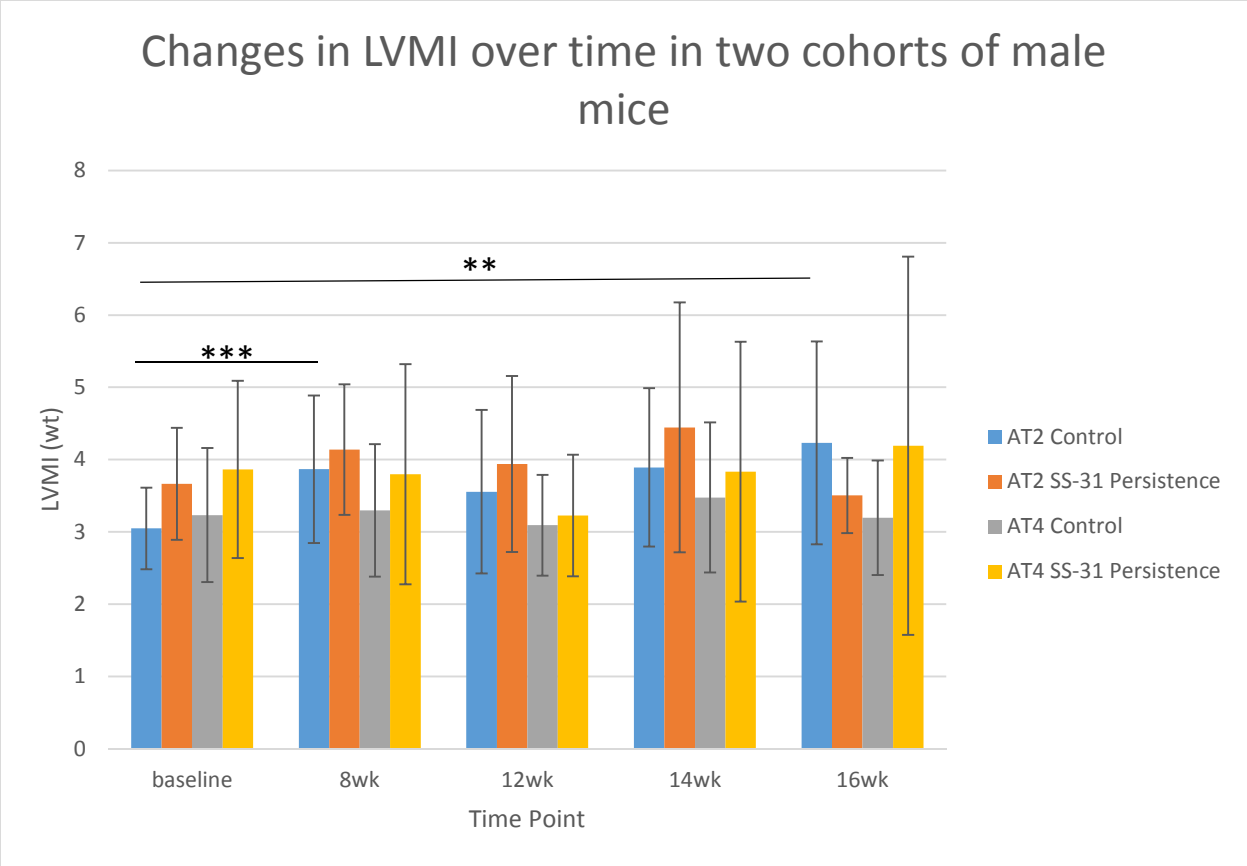


Figure 36: Average changes seen in two male cohorts of mice for LVMI (wt). There are no significant changes seen in AT4. In the AT2 cohort, there is a significant increase LVMI (wt) by paired t-test comparing the baseline control group to the 8 week and the 16 week time points (**=p<0.01, ***=p<0.001) (AT2 n=10-11; AT4 n=10-12). Error bars indicated standard deviation.

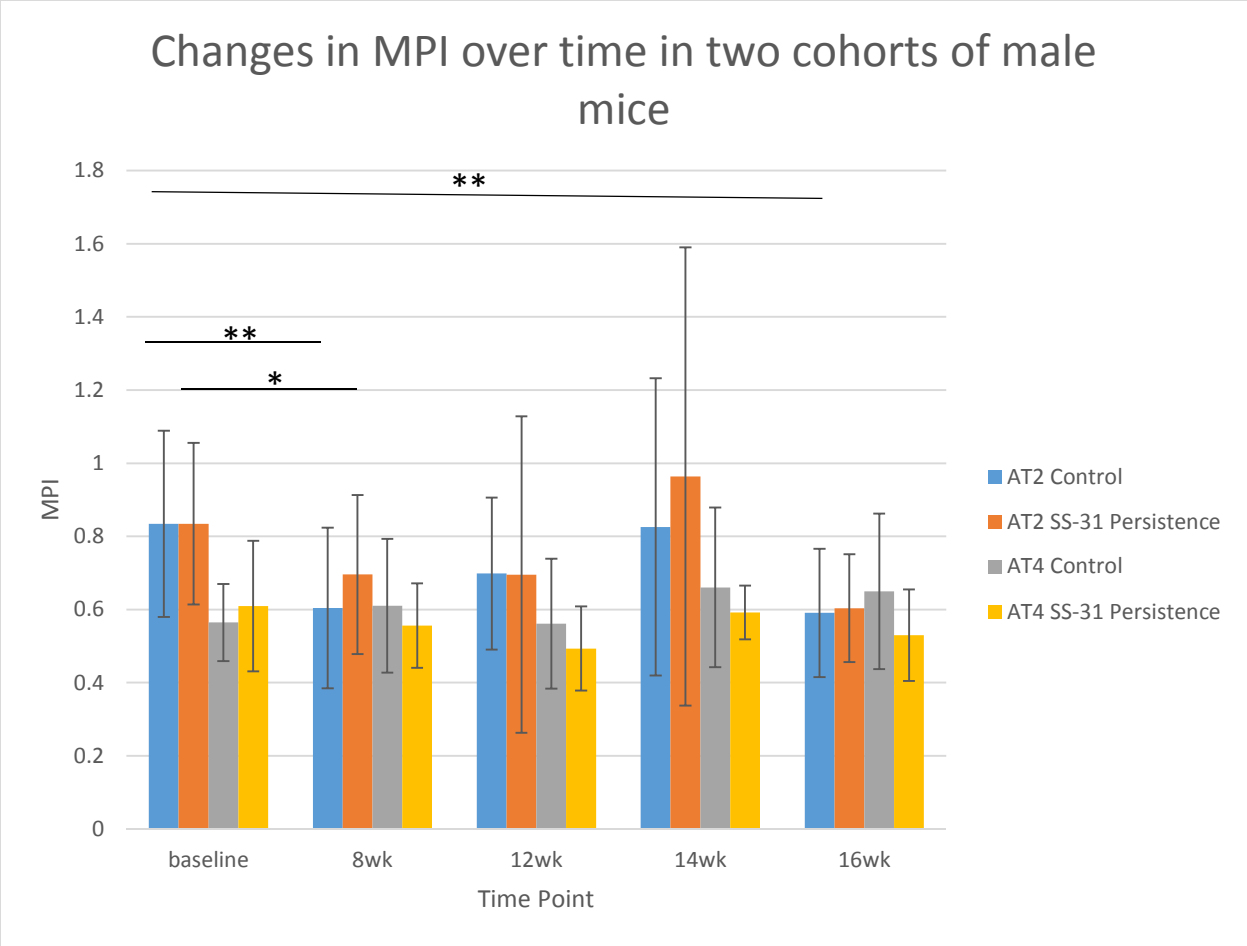


Figure 37: Changes in MPI measurements via echocardiography for two cohorts of male mice during SS-31 persistence. In AT2, we see a significant decrease in MPI between the baseline and 8 week measurement for both the control and the SS-31 persistence group by paired t-test (*=$p<0.05$, **=$p<0.01$). Additionally, using the same analysis, we also see a decrease between baseline and the 16 week time point in the control mice. No significant changes were seen in AT4 (AT2 n=10-11; AT4 n=10-12). Error bars indicated standard deviation.

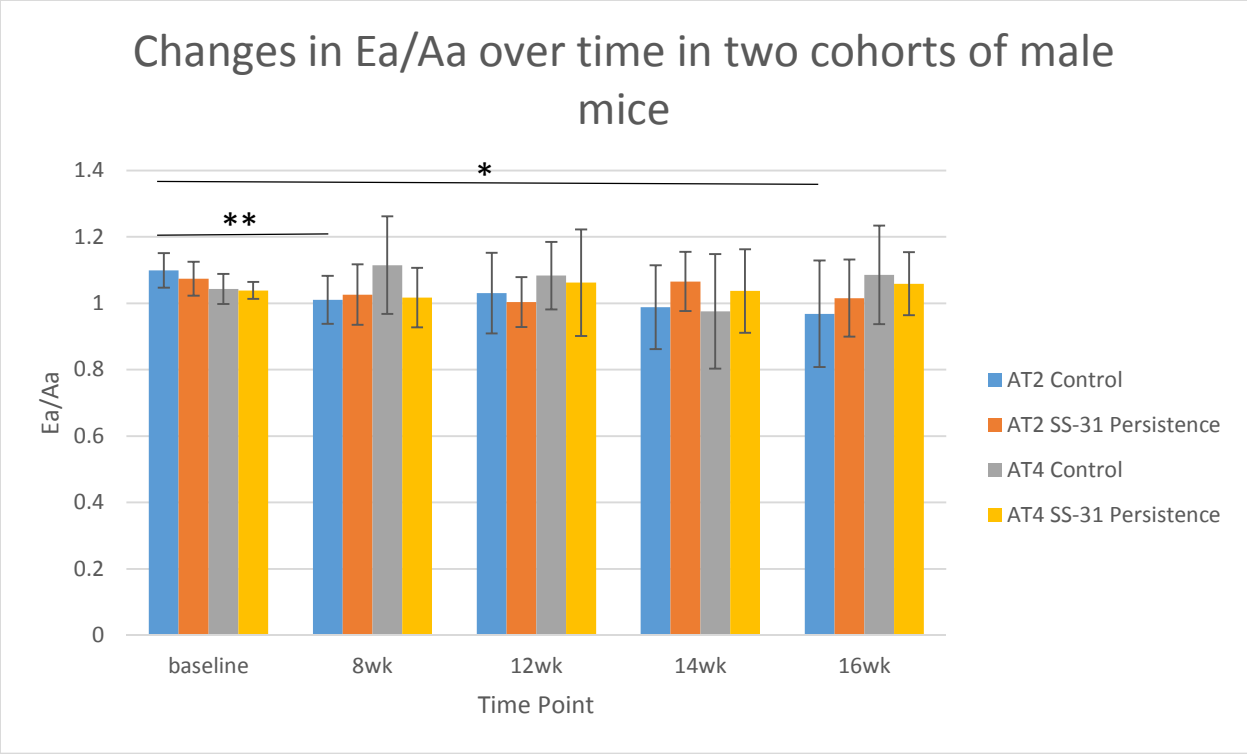


Figure 38: Changes in Ea/Aa measurements via echocardiography for two cohorts of male mice. In the first male cohort, AT2, there was a significant decrease by paired t-test compared to baseline in both the 8 week and 16 week time points (*=p<0.05, **=p<0.01) (AT2 n=10-11; AT4 n=10-12). Error bars indicated standard deviation.

Unlike the 8 week treatment data, there are many more similarities between the male and female cohorts in the SS-31 persistence studies. It can be noted that again, the male mice have more changes in their ejection fraction, fractional shortening, and LVMI compared to the females, and the females exhibit more changes in the MP and E'/A'. These general changes might suggest different, gender based changes, but due to the complications within the experiment, it is not possible to determine if there is a true gender difference. Future studies could be carried out to more thoroughly investigate gender based differences.

In the previous experiments we saw an improvement in the MPI and E'/A' function of SS-31 treated mice, in this data, we saw a similar occurrence that continued at 10 weeks, but lost persistence by 16 weeks, suggesting the functional effects that SS-31 provides a continued effect for at least 2 weeks after drug removal, but decline thereafter. In contrast, the E'/A' ratio steadily declined in both groups, in contrast to the previous studies. This suggests that there is no continued persistence in the functional improvements of SS-31. Overall, there are no changes in either gender when in LVMI, ejection fraction, or fractional shortening, suggesting that SS-31 did not have a strong influence on these components.

Within these findings there are some caveats to this data. Echocardiography is an inexact science that takes many hours of practice before a true level of expertise is reached. The quality of echocardiograms improved over the time courses, but the initial study in the female mice was less than ideal. In the AT2 cohort, the author experienced health complications that could affect the quality of data obtained. The overall standard deviation was higher in all cohorts compared to previous studies. Finally, previous

studies done had analysis done when the researchers knew the treatment for each mouse, while the mice in the male cohorts were blinded for data acquisition and data analysis in an attempt to remove any possible researcher bias. In all, it is possible to look further into comparisons that approach significance ($0.05<p<0.1$), of which there were twenty.

Heart weight measurements were taken at sacrifice time. Heart weight (mg) was normalized to body weight (g) in all three cohorts as can be seen in figures 39 and 41. Additionally, in the female cohort, hypertrophy was measured by taking the heart weight (mg) over the tibia length (mm) as can be seen in figure 40. Regardless of gender or the way that hypertrophy was measured; there was no significant change between groups by unpaired t-test.

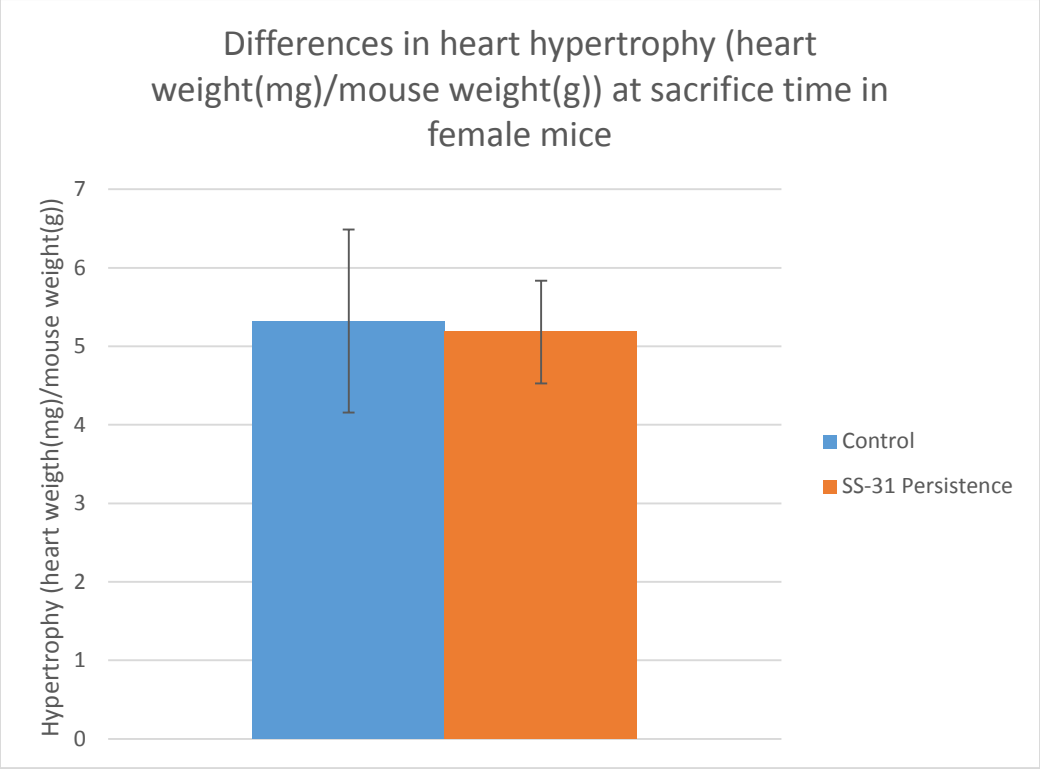


Figure 39: Measurement of hypertrophy from heart weight (mg) over body weight (g) at sacrifice time for female mice. There was no significant difference by unpaired t-test (n=6-9). Error bars indicated standard deviation.

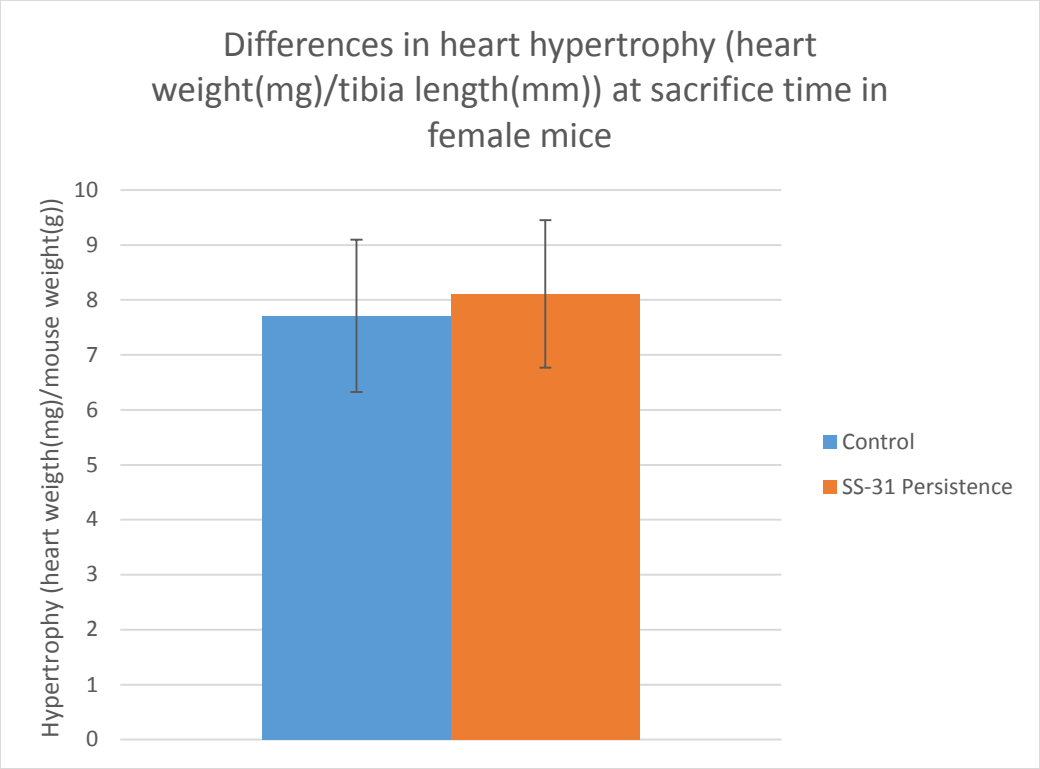


Figure 40: Measurement of hypertrophy from heart weight (mg) over tibia length (mm) at sacrifice time for female mice. There was no significant change by unpaired t-test (n=6-9). Error bars indicated standard deviation.

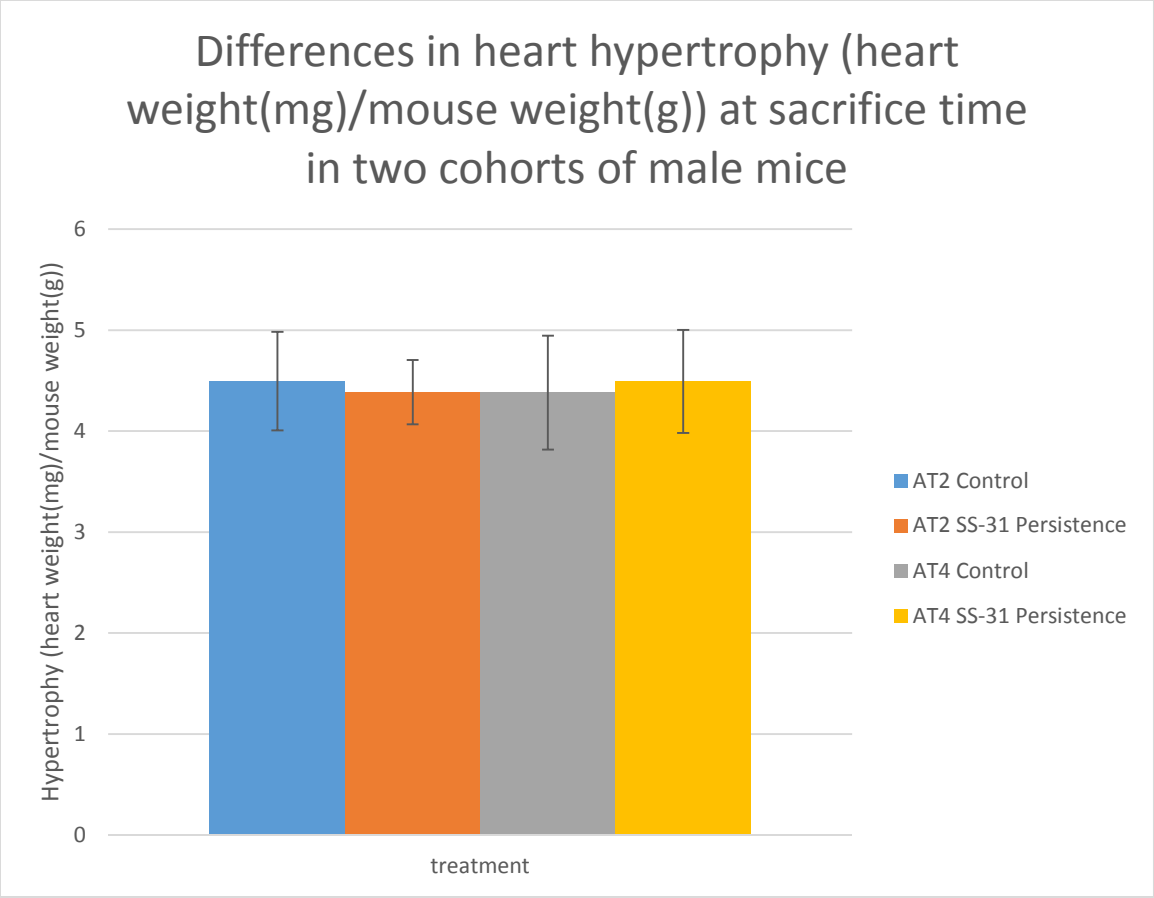


Figure 41: Measurement of hypertrophy from heart weight (mg) over body weight (g) at sacrifice time for male mice. Neither cohort was significant by unpaired t-test (AT2 n=10-11; AT4 n=10-12). Error bars indicated standard deviation.

V. In Vivo Studies (treadmill & grip strength)

Work performed by Dr. Marcinek's lab has shown that SS-31 treatment improves mice running times on a treadmill. With this knowledge, we decided to investigate if this phenomenon is continued in the SS-31 persistent mice. Mice were run on a mouse treadmill (Eco 3/6; Columbus Instruments, Columbus, OH, USA), after an acclimation evening, during their awake cycle at baseline, eight, and sixteen weeks. Running times were recorded and running distances calculated.

As can be seen in Figure 42 and 43 below, overall running distance did not improve either the female or male cohorts. Additionally, the male mice at 16 weeks were unable to complete the first two minutes of the ramping protocol. This led to no meaningful data and is not included. After completing the studies on treadmill running distance, power analysis were completed to investigate the number of mice would need to be used to see a statistical difference with the current difference in our groups. From that analysis, we were able to see that we would need to more than quadruple the number of mice used. Due to the limitations of this large number, it was decided to look into another measurement of muscle strength, as opposed to endurance.

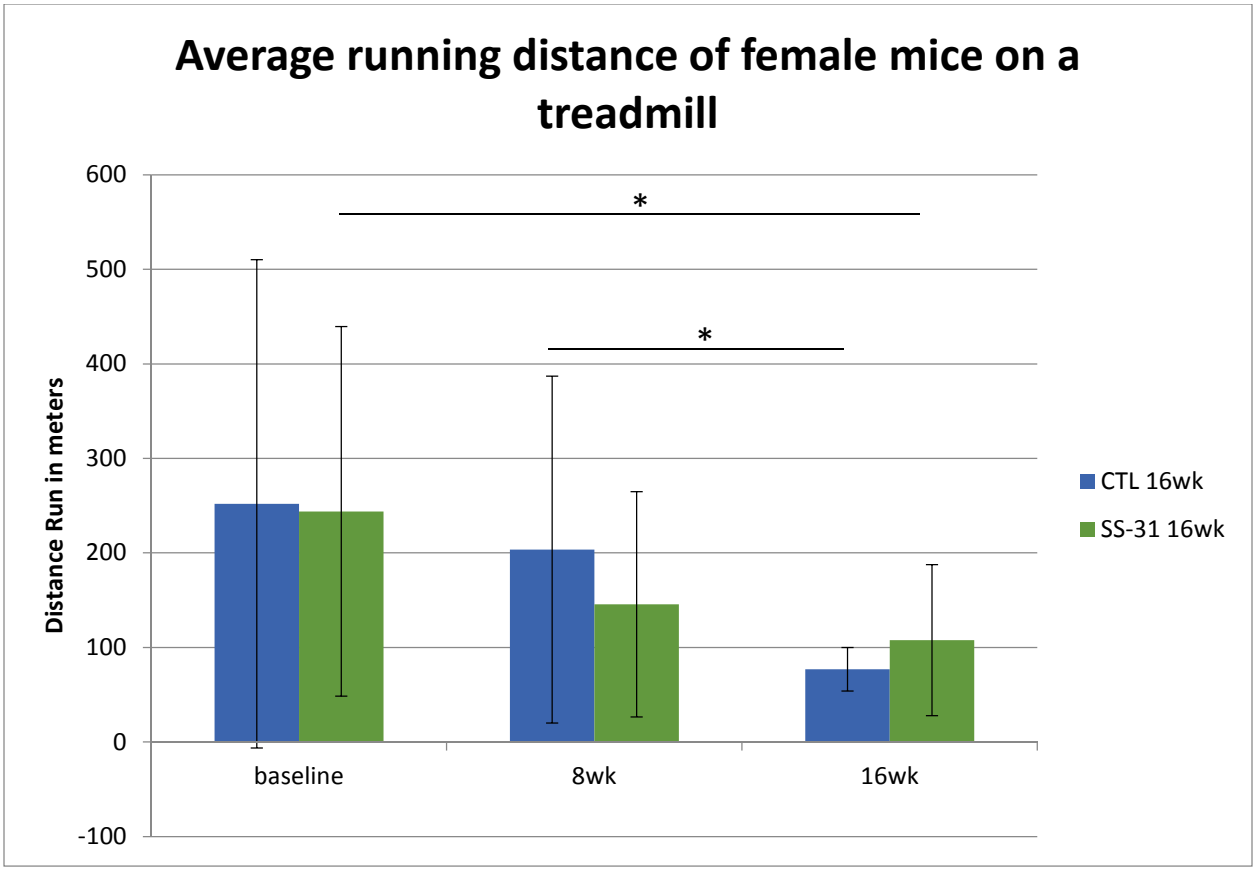


Figure 42: Changes in treadmill running distance in female mice. Overall, there was no noticeable improvement of the SS-31 treated mice compared to the control treated mice. However, the distance run by the control mice was significantly decreased at 16 weeks compared to the 8 week time point. Additionally, the distance the mice ran was significantly decreased at the 16 week time point compared to the baseline time point (*=$p<0.05$) (n=6-9). Error bars indicated standard deviation.

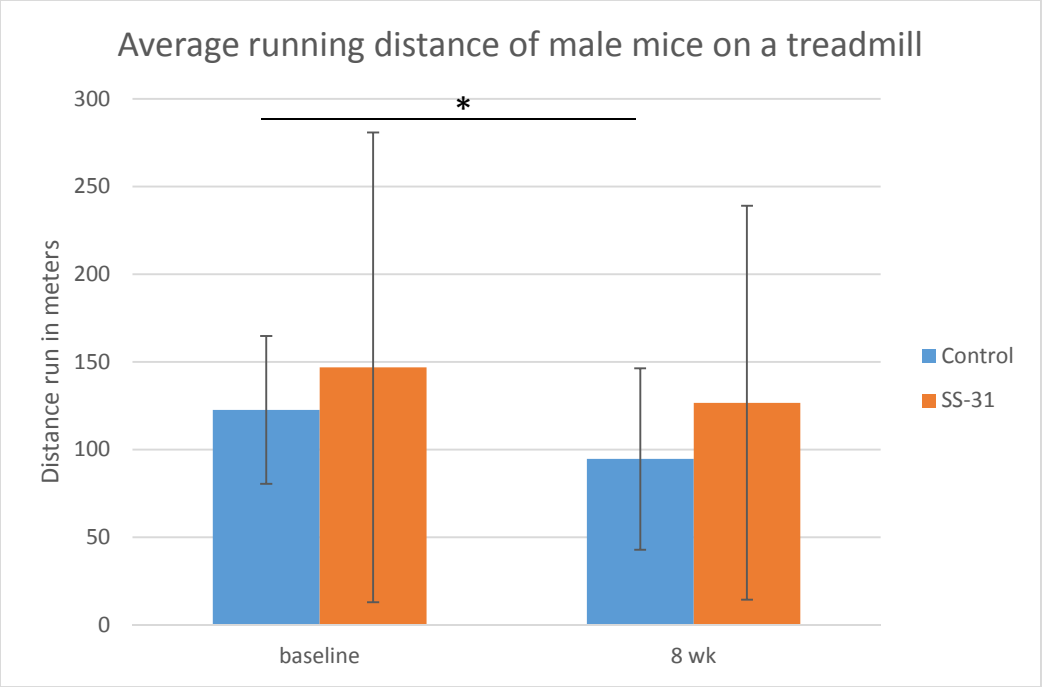


Figure 43: Change in distance run on a mouse treadmill in male mice. The general trend, saw a decrease in distance run, however, there was no significant change between the groups at either time point. There was a significant decrease in running distance from baseline to 8 weeks of treatment in the control mice (*=p<0.05). No 16 week data is available because the mice did not run (n=13-15). Error bars indicated standard deviation.

Grip strength was investigated in a cohort of male mice to see if there were any changes in this measurement compared to muscle endurance. Mice were given five trials to grip the GRIP STRENGTH apparatus with PC interface (San Diego Instruments, Inc.), with their highest strength recorded at baseline, eight, and sixteen weeks.

While there were some significant changes seen across time points (Figure 44), as can be seen in the decrease in strength at eight weeks in both groups compared to their own baseline, and the increase at 16 weeks compared to 8 weeks for the SS-31 persistent mice, there were no significant changes between the two treatments at any time point. However, with the large standard deviations, and the difficulty in ensuring each mouse got a strong starting grip, it is possible to see some questions in the data. For example, if the n were larger, or the standard deviation smaller, we might have seen a significant increase in grip strength at the 16 week time point. Currently, it is possible to say that it appears that there is a trend towards an increase in grip strength at this time point for the SS-31 persistent mice.

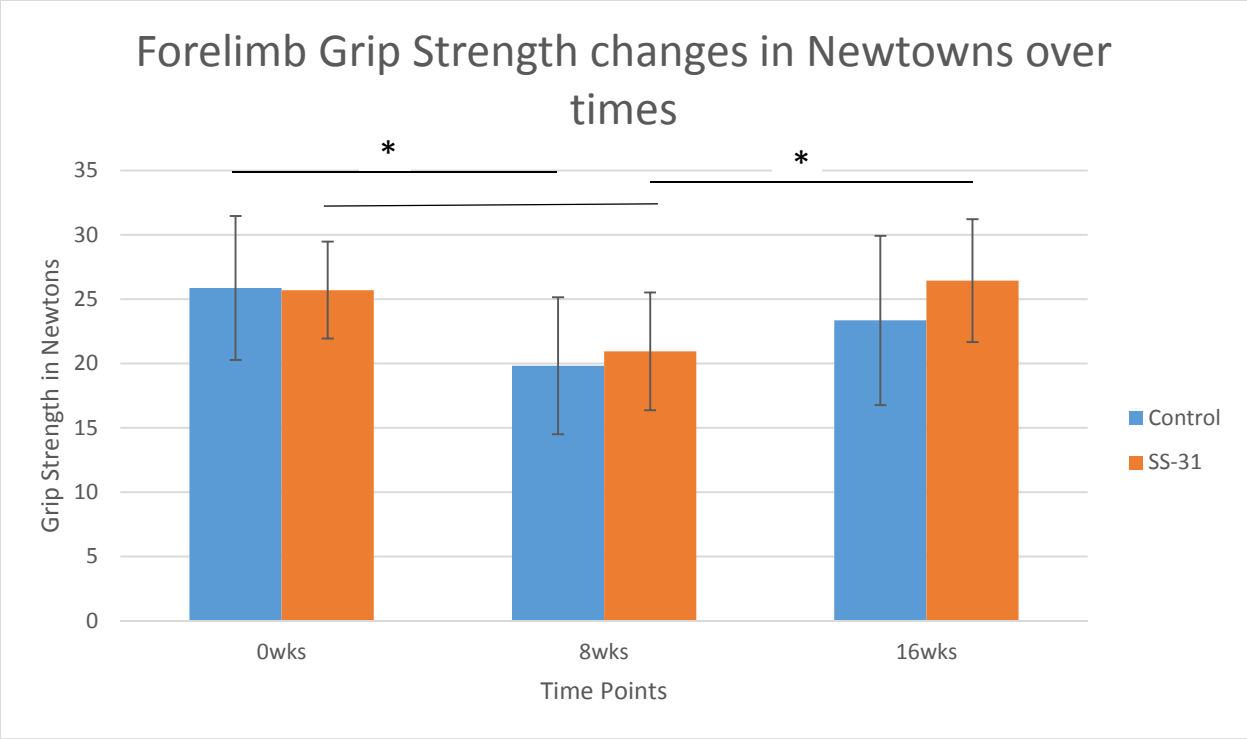


Figure 44: Mouse forelimb grip strength measurements at baseline, eight, and sixteen weeks. There is a significant difference between the grip strength of the SS-31 treated mice when compared to the next time point in sequence via paired t-test, but there was no change in from baseline to sixteen weeks ($p<0.05$). Grip strength significantly decreased for control mice from baseline to eight weeks in a paired t-test ($p<0.05$). No other significant changes were seen. (n=10-15). Error bars indicated standard deviation.

II. Ex Vivo Assessments

a. Proteomic changes by mass spectroscopy

As already detailed, heart tissue was powdered to prepare for multiple analysis. Proteomics by mass spectroscopy was performed. Proteins were extracted from frozen heart tissues in ammonium acetate with rapigest, and then were trypsin digested and purified by MCX column (Waters). LC-MS/MS analysis was performed with a Waters nanoAcquity UPLC and a Thermo Scientific LTQ Orbitrap Velos, and the MS analysis of protein abundance was recorded. This was detailed in (Dai et al., 2014). Below we see some of the comparisons between the young, old, and persistence groups.

In figure 45, we see the peptides z-score for the female and male cohort of mice. In both cohorts, the time point that the samples were taken at (either 8 weeks or 16 weeks) shared more similarities and changes in the same direction than the treatment groups compared to each other. Additionally, compared to the combined therapy data, the direction of change was similar for the treatment groups between the two genders. For example, in the mitochondrial dysfunction groups, the 8 week treatment groups had a decrease, relative to z-scores, in their proteins, while the 16 weeks saw an increase,

regardless of gender. However, even within this same group, there are unique differences in the peptides between the genders, suggesting that there are proteomic differences between the genders.

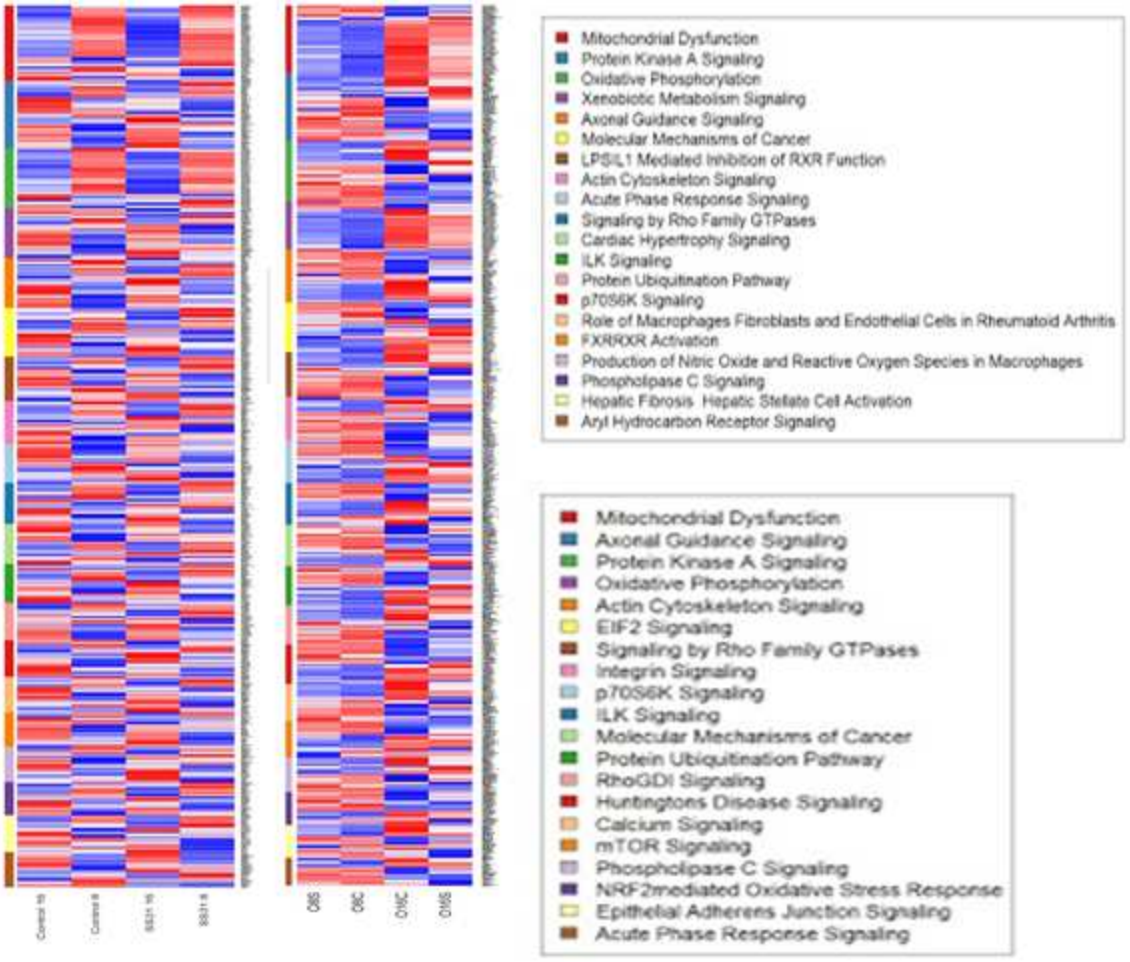


Figure 45: Proteomic analysis by IPA pathways for AT1 (left) and AT2 (right) with their labeled, color coordinated pathways on the upper right and lower right respectively. Multiple pathways show different changes in the pathways, but no defined clear cut relationship is visible (n=3-11).

For a better insight into proteomic changes, figure 46 was used to investigate the top six IPA pathways in each gender. The female cohort is on the left hand side of the figure, and the male cohort on the right side of the figure, with their top six pathways listed below them. Similar to the combined therapy groups, the two genders share four of the six top IPA pathways in common, including mitochondrial dysfunction, protein kinase A signaling, axonal guidance signaling, and oxidative phosphorylation. Additionally, the unique pathways are the same as the combined therapy, with female mice showing xenobiotic metabolism signaling and molecular mechanisms of cancer within their top 6 pathways, and male mice containing actin cytoskeleton signaling and EIF2 signaling in their top 6 pathways. Again, it should be noted that this does not mean that the opposite gender does not exhibit these pathways or their changes, only that they might occur later on in the IPA pathway analysis. Unlike what was seen in the young mice, it does appear that the change in z-score is not the complete opposite of each other in

their respective genes. For example, in mitochondrial dysfunction, both 8 week treatments begin by showing an increase relative to their z-scores, but after some of the proteins there is a reverse in this signature. This suggests that some of the changes that we observe are similar in both genders. A complication of this, however, is the fact that the proteins may not be in the same order. While these zoomed in images continue to show that the most common separation is by age, it does also show some locations where the changes are due to treatment and not age, suggesting that SS-31 does have some effect, albeit small compared to age, in the persistence treated mice.

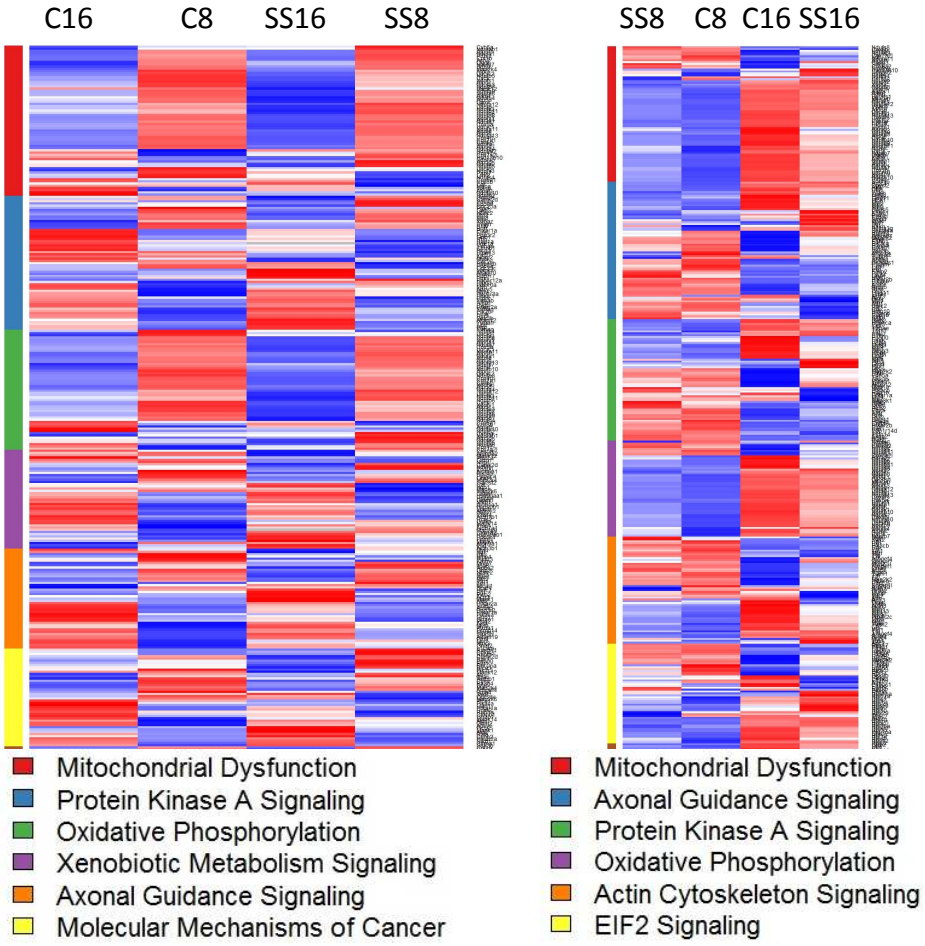


Figure 46: AT1 and AT2 magnification of the top part of the IPA pathway sorted images. The protein categories vary between the female and male treated groups, as can be seen by the colored groups and the respective legends for each group. Again, we see that the groups are most strongly separated by age, not by treatment group (n=3-11).

While mass spectroscopy across IPA pathways is informative in the overall processes that vary, they can prove difficult in showing a relationship with changes between the groups. Part of this complication is that the pathways often have repeating peptides between the pathways, sometimes showing multiple large differences that in reality only represent one large change. To help define these differences, figure 47 separates the groups by their similarities in the form of relationships of divergence, including young animals to see proteomic changes between young and old mice. In the female mice, there are two unique separations. One of these separations has the two 16 week treated mice together, suggesting

that their proteomic profile is the most similar to each other and that they are the most different to the 8 week treated and young mice. In the other group, the young mice were more similar to each other than they were to the 8 week treatment groups. Within the male cohort, the same changes were seen as in the female cohort with the 16 week treatment groups belonging to a group outside the 8 week treatment and young group. These findings suggest that the greatest effect on the proteomic changes is not by the SS-31 peptide that the mice received, but rather by the aging process itself. Additionally, there appears to be a large proteomic shift in mice from 26 months to 28 months, making conclusions about the therapy more difficult to conclude and will suggest a larger cohort will be necessary to assess the role of the peptide vs. the role in changes.

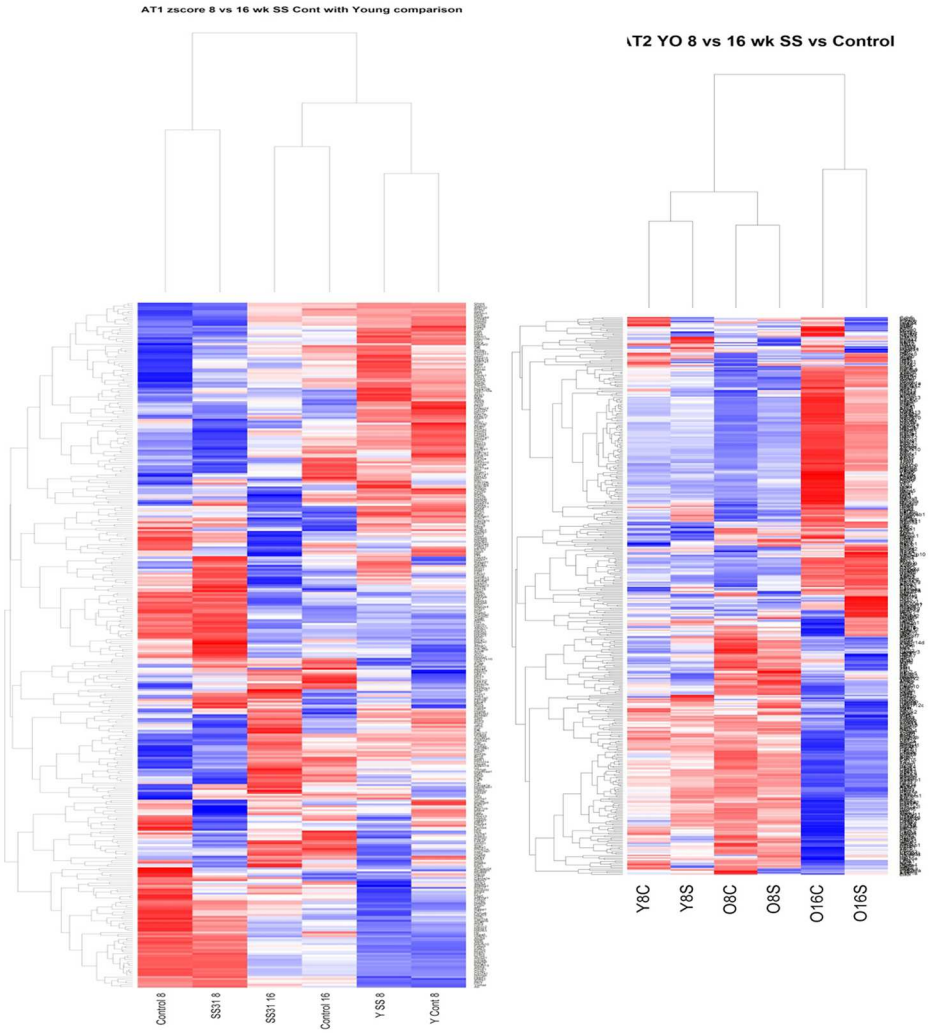


Figure 47: Mass spectroscopy analysis of cardiac peptides from a cohort of male and female mice by z-score for relationships of change between various treatment groups. Time points are more similar to each other than treatment groups are to each other (n=3-11).

Proteomics is a great tool to investigate changes that occur across the whole proteome, but as discussed before, there are still challenges that arrive from these methods. In addition to the loss of insoluble

proteins, there is the challenge of the exact detail a mass spectrometer can give about a sample. While this machine is great, there is still a threshold on the amount of peptides that can picked up at one time, possibly losing peptides that might be changing, but only do so to a small degree. This is a constant challenge, and one that individuals working exclusively in this field are trying to overcome. Overall, there was a good n for these two cohorts of mice, but it appears that the largest challenge within this cohort was the age of the mice. It appears that there is a large proteomic shift in the oldest group of mice, suggesting that a younger starting time might be necessary to elucidate the role of the peptide.

a. Protein transcription changes by qPCR

To measure changes in the transcriptional levels of proteins, quantitative PCR was performed. Powdered heart sample was used to isolate RNA and made into cDNA. cDNA was used with a standard curve to calculate the amount of protein transcription. Prior qPCR experiments in the lab for SS-31 investigated markers for biogenesis, which is detailed below for the SS-31 persistence study.

Within the first group of qPCR measurements was that of myoglobin, figure 48. Myoglobin is not been linked to changes in aging in the cardiac tissue, making it a good candidate for normalization. As can be seen in figure L below, there is no statistical difference between any of the treatment groups, marking myoglobin as a good normalizing factor.

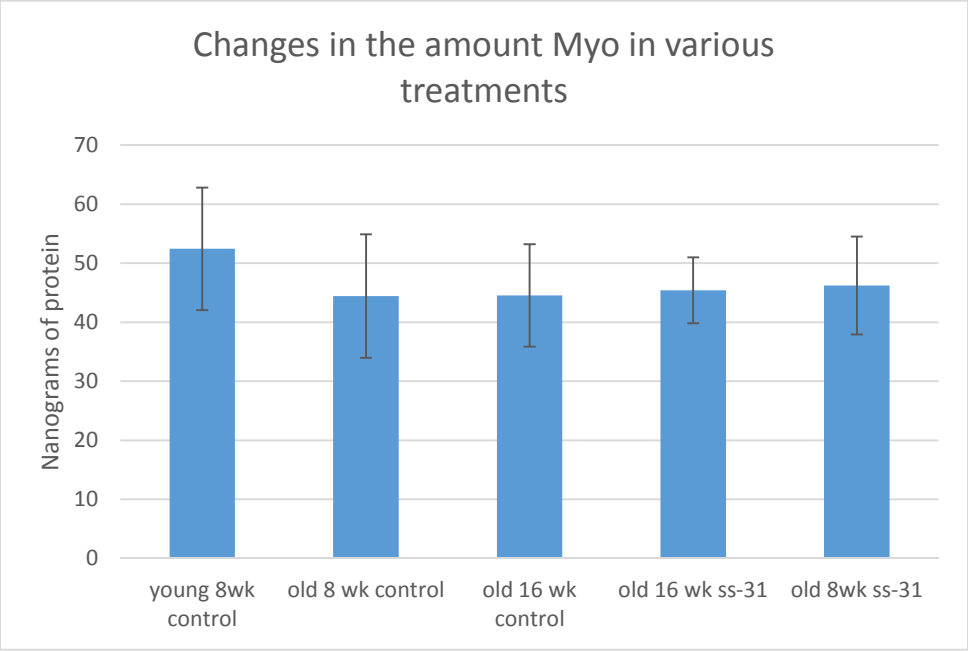


Figure 48: Measurement of Myo in nanograms across various treatments to be used as a normalizing value (n= 4-6). Error bars indicated standard deviation.

The first transcript that we investigated was PGC-1α. PGC-1α is recognized as being the main assimilator for biogenesis (Boland et al., 2013). Downstream of this transcript we see many more of the proteins often recognized in mitochondrial biogenesis, including ESRRA and TFAM. In our qPCR data, we see no significant changes between any groups when normalized to myo as can be seen in figure 49.

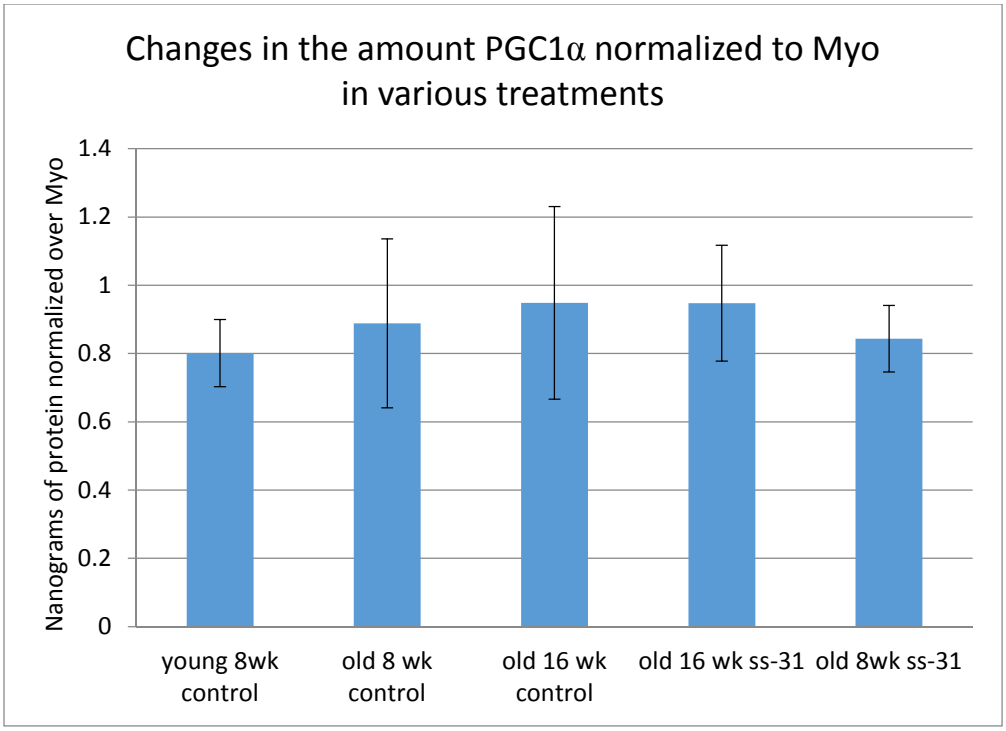


Figure 49: Measurement of change in PGC1α transcription normalized to Myo across all treatment groups. There was no statistical change between any of the groups (n= 4-6). Error bars indicated standard deviation.

While this data might suggest there are no changes in biogenesis, there are other activators of biogenesis. Keeping this in mind, we looked at the downstream targets of PGC-1α that are still involved in biogenesis. ESRRA, or ERRα, is another protein found in the biogenesis pathway. Unlike PGC-1α, we do see a significant increase in its transcript (Figure 50). Specifically, there is an increase compared to baseline in the 16wk control, 16 week SS-31, and the 8 week SS-31 treated groups. This suggests that there is an increase in expression with the SS-31 8 week treated mice, and an aging effect on the mice at 28 months, since there is no significant difference between the two 16 week groups. Additionally, the 8 week SS-31 treated group is significantly increased compared to the 8 week control group, suggesting that the 8 week treatment has an effect on the transcription of ESRRA.

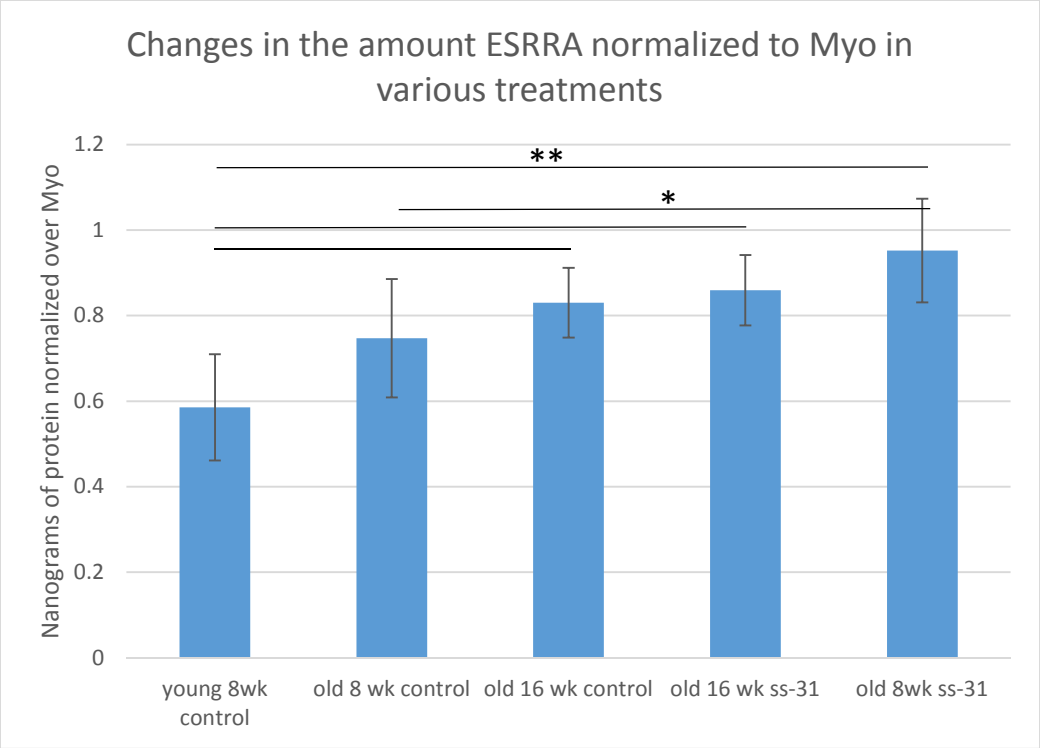


Figure 50: Measurement of the change in ESRRA transcription normalized to Myo across all treatment groups. There is a significant increase in the 16 week SS-31 treated and 16 week control compared to the young control group. Additionally, the 8 week SS-31 treated group was increased compared to the either the young control or the old control group (*=p<0.05, **=p<0.01) (n= 4-6). Error bars indicated standard deviation.

The final mitochondrial biogenesis marker we looked at was TFAM. TFAM is down stream of ESRRA, with many other targets. In figure 51, we see some significant changes that are more difficult to understand than the ESRRA changes. Like ESRRA, there are significant changes between the young control mouse and the older mice; however there is not a clear cut age different. Compared to young mice, both the 8 week SS-31 treated group and the 16 week control group show a significant increase in their TFAM expression. There is also a significant increase in TFAM expression in the 8 week SS-31 treated mice compared to the SS-31 persistent mice, suggesting that the effect that SS-31 does have is not persistent. Additionally, these data suggest that there is no real change between the older mouse treatment groups, compared to each other, giving no real evidence that SS-31 has any observable effect on TFAM in the biogenesis pathway.

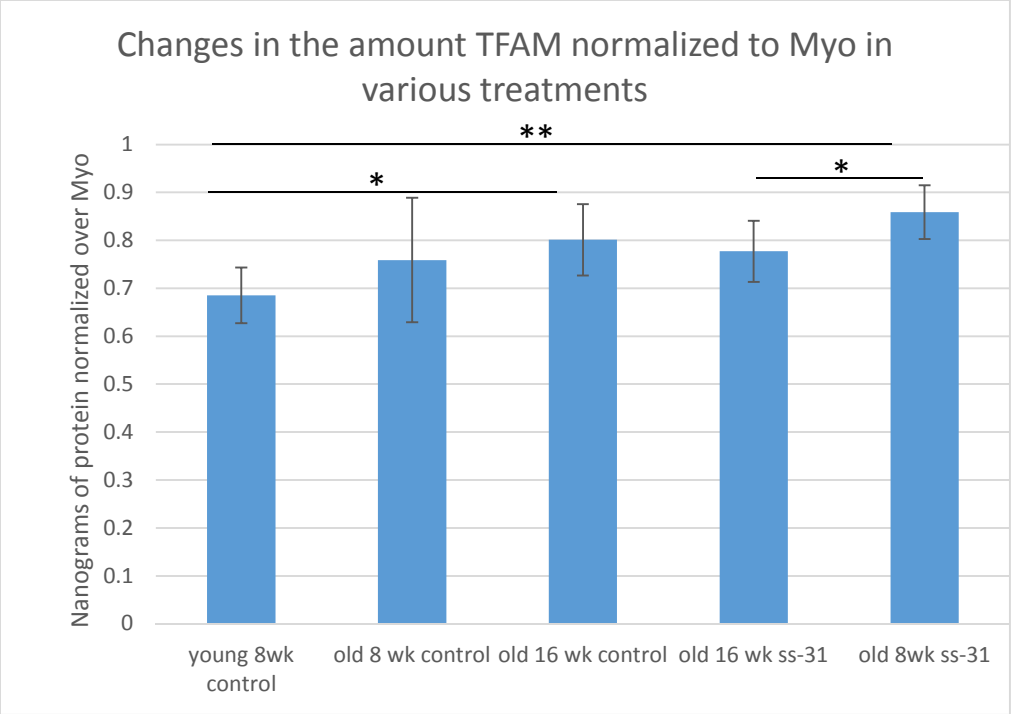


Figure 51: Measurement of change in TFAM transcription normalized to Myo across all treatment groups. The 8 week SS-31 treated group was significantly increased compared to either the young control or the 16 week SS-31 treated group. Additionally, the 16 week control mice had a significant increase compared to the young control mice (*=p<0.05, **=p<0.01) (n= 4-6). Error bars indicated standard deviation.

Overall, there were some changes in the biogenesis pathway in the three markers we investigated, but none of them suggest a strong effect on SS-31 treatment or persistence. The largest effect seen in biogenesis transaction expression is between the young control mice and the old mice, suggesting that the largest changes seen as a change in age. Looking into proteomics data could help us find better transcriptional targets, by noticing large changes, but additional biological samples could help increase the statistical power, giving more insight as well. Finally, all the qPCR data was performed from the male cohorts of mice. Keeping in mind the possible differences between male and female mice in other aspects of this persistence, investigating the expression levels in female mice might highlight different changes and roles for these proteins.

b. Protein levels by Western Blot

To investigate the protein levels, some of the powdered heart tissue was placed into lysis buffer where proteins were extracted. Those protein samples were then run on gels and stained with antibodies to look at protein levels. Five different antibodies were run on the blots, with each individual band being normalized to the whole protein stain for its sample, and then normalized to changes in protein level compared to young control.

To begin investigating the protein levels by western blot, we start with the oxidative phosphorylation cocktail that shows a protein from every subunit of the mitochondrial oxidative phosphorylation change.

While there are few significant changes in any of the complexes, the overall changes that we can see help explain the changes in the proteomic analysis.

When looking at the NDUFB8 data (figure 52), there is again seen a trend towards a decrease in the 8 week treated SS-31 treated group, this trend is seen in the persistence group as well. However, there appears to be a greater degree of similarity between the persistence groups with each other than with the 8 week treated groups with each other. This suggests that if SS-31 had any effect, it is mostly gone by the time point 8 weeks post-drug removal, and that the mice at 28 months of age appear to have similar levels of NDUFB8. The decrease in this protein, suggests a decrease in the efficiency of complex I which would decrease efficient energy production and possible create more reactive oxygen species.

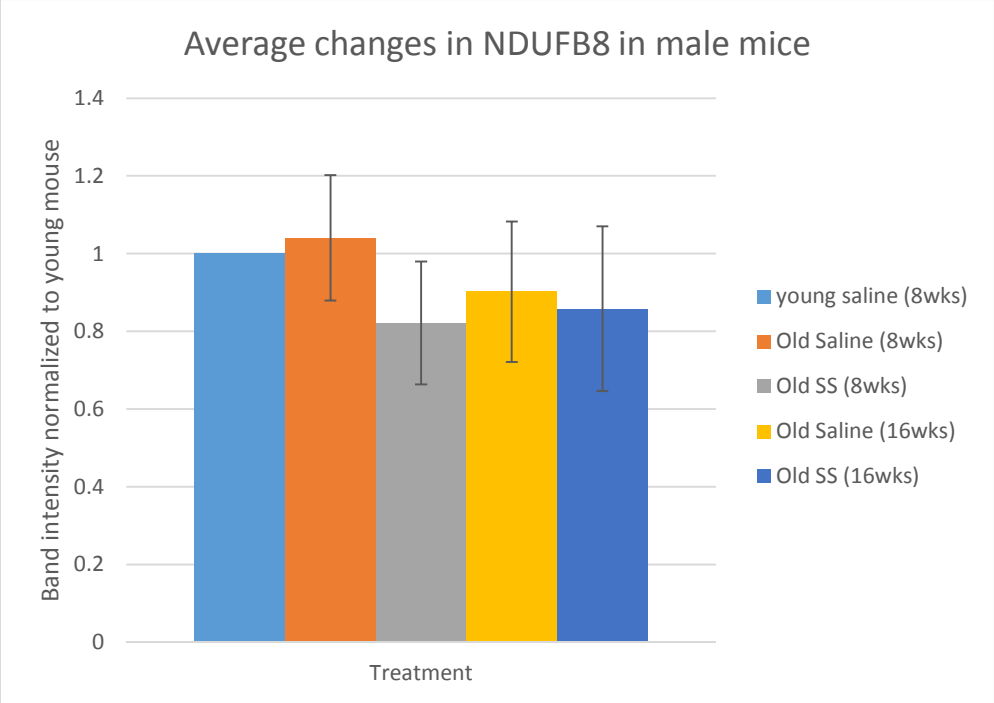


Figure 52: Average changes seen in male mice in oxidative phosphorylation protein NDUFB8 from complex I. There is no significant change between any groups (n=5). Error bars indicated standard deviation.

The changes observed in the protein levels of SDHB in the persistent mice are not significant, as can be seen in figure 53. There are no great trends to be seen within the data set, with the exception that mice receiving SS-31 had a slightly increased level of SDHB compared to their aged match controls. This might suggest that SS-31 helps maintain the level of SDHB as the mice age, but there is no great difference in protein levels between the young and old mice.

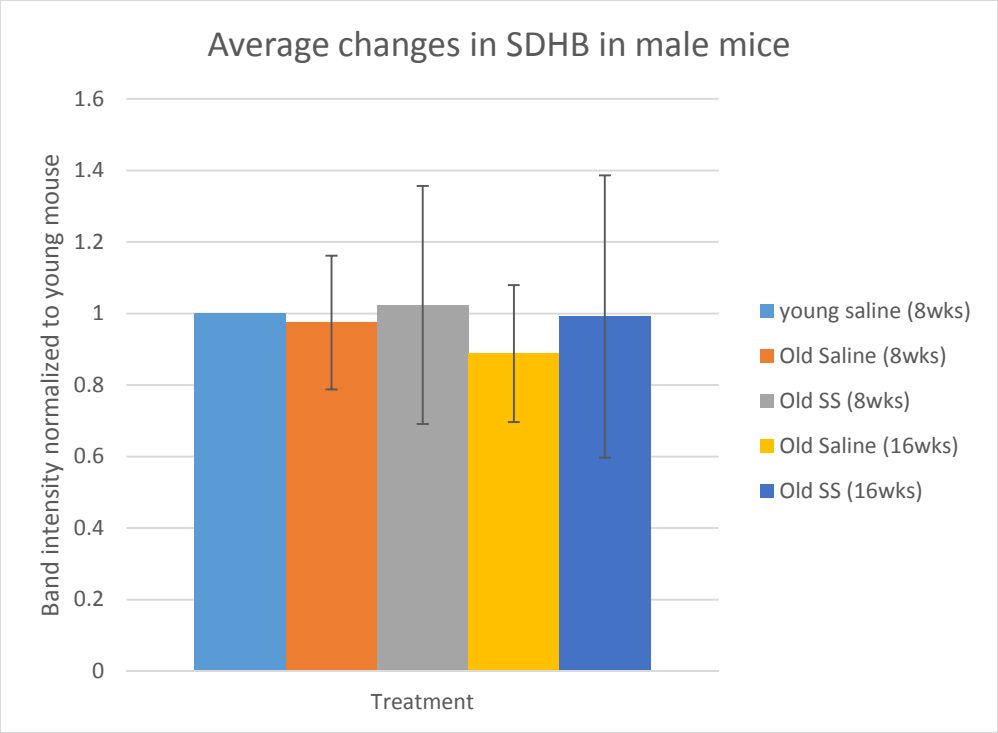


Figure 53: Average changes seen in male mice in oxidative phosphorylation protein SDHB from complex II. There is no significant change between any groups (n=5). Error bars indicated standard deviation.

In the UQCRC2 protein of complex III, there was only one significant change (figure 54). There was a significant decrease of UQCRC2 in the 16 week saline treated mice compared to the young control. However, in general, there appeared to be a trend towards a decrease in UQCRC2 protein levels in all the old treated groups, suggesting that UQCRC2 is naturally reduced as mice age. Additionally, in the SS-31 groups, whether treated or persistent, it appears that the decrease was lessened compared to the age matched saline controls. This indicates that SS-31 might have a positive effect on the level of UQCRC2, whether while treated or after treatment is removed.

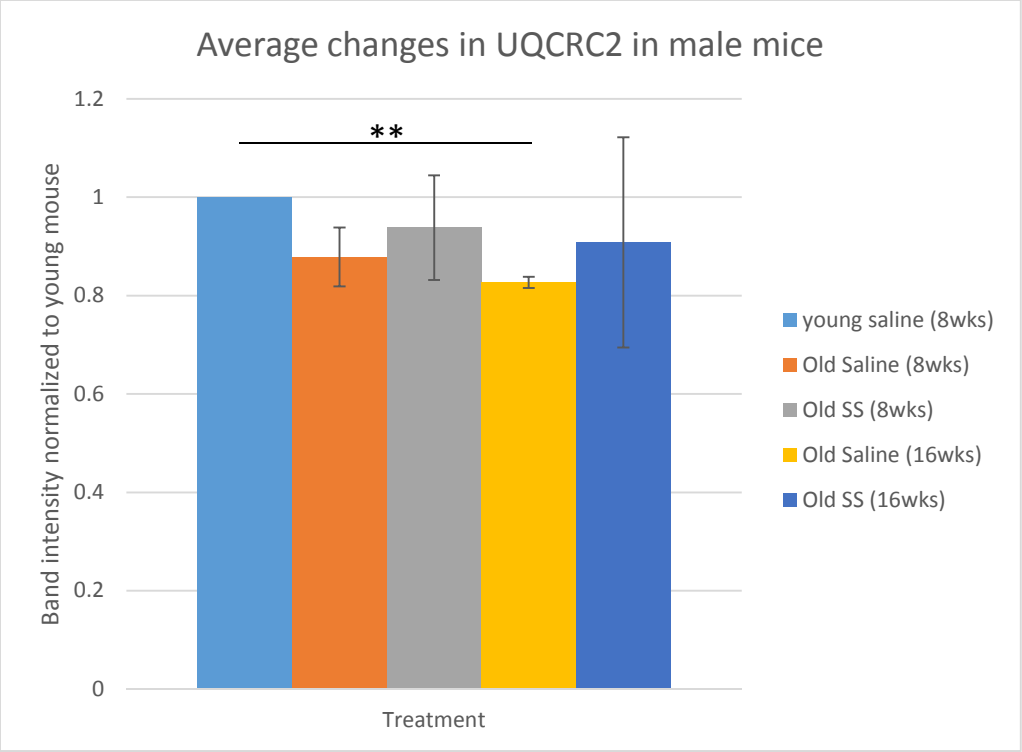


Figure 54: Average changes seen in male mice in oxidative phosphorylation protein UQCRC2 from complex III. There is a significant decrease in 16 week saline treated mice compared to the young control (**=p<0.01) (n=5). Error bars indicated standard deviation.

In figure 55, the changes in the level of MTCO2 are observed in the various groups for the persistence experiments. Within these groups there is no significant change. Overall, the standard deviations are large for all samples, making conclusions challenging. It is interesting to note that there appears to be an increase in expression for the SS-31 treated mice at 8 weeks and the saline treated mice at 16 weeks. Additionally, the SS-31 persistence mice have the lowest levels of MTCO2. While the increases are difficult to explain in the two different groups, it is possible that the decrease in the SS-31 persistence group is that the effect that was occurring at 8 weeks has a larger effect on its loss than a continued decline in an aging model. Future studies will have to explain this strange interaction.

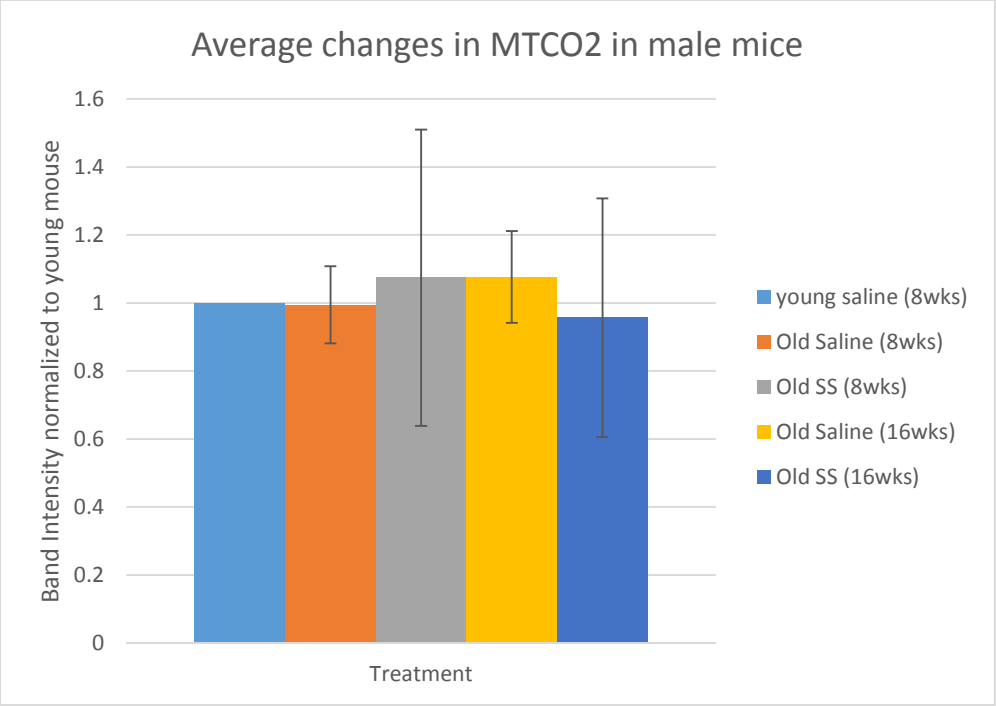


Figure 55: Average changes seen in male mice in oxidative phosphorylation protein MTCO2 or UQCRC2 from complex IV. There is no significant change between any groups (n=5). Error bars indicated standard deviation.

The final protein that we investigate is ATP5A in complex V of the electron transport chain. In figure 56, there are no significant changes for any of the groups. Generally, all groups appear to be increased compared to the young control group, except for the SS-31 persistence group. This suggests that there might need to be an increase in the protein levels for oxidative phosphorylation to be completed efficiently. The steady level in the SS-31 persistence group suggests that there is adequate ADP to ATP conversion occurring, but no extra protein is needed.

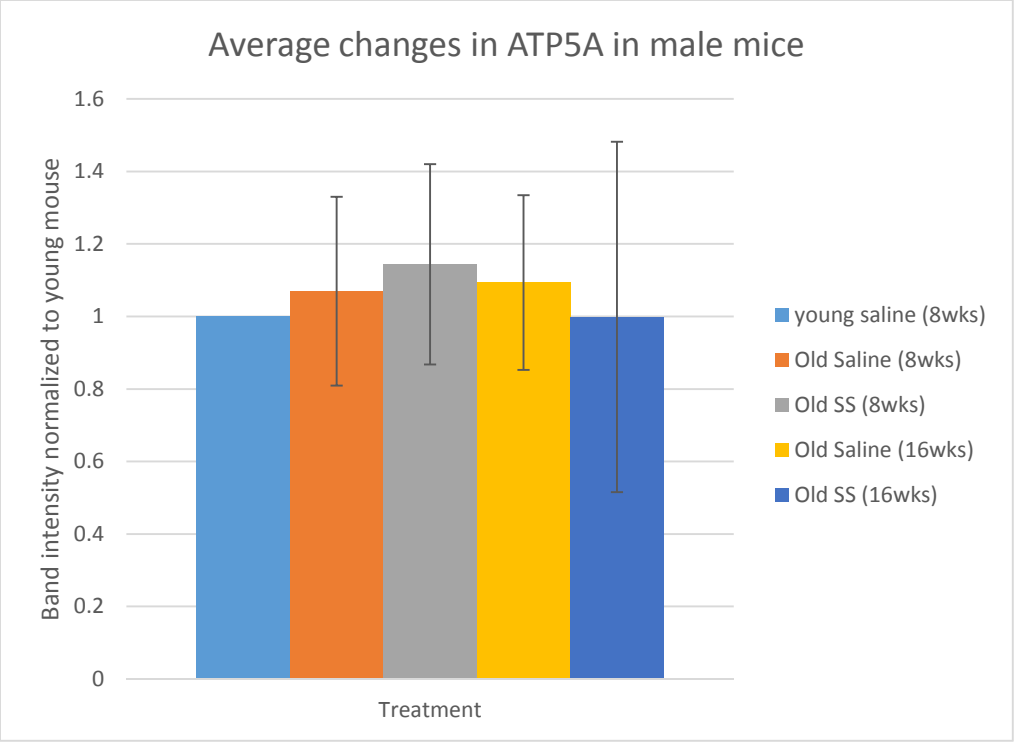


Figure 56: Average changes seen in male mice in oxidative phosphorylation protein ATP5A from complex V. There is no significant change between any groups (n=5). Error bars indicated standard deviation.

Measuring changes in autophagy by LC3II/I ratio shows the same change as the combined therapy in the form of a significant decrease in the SS-31 treated mice at eight weeks (Figure 57). However, there are no other significant changes. There is a trend towards an increase in LC3II/I in the two 28 month groups, possibly suggesting another specific age change, since it appears that the 26 month old mice trend towards a decrease in LC3II/I. However, the data is not conclusive and future work will need to discover if these results are maintained.

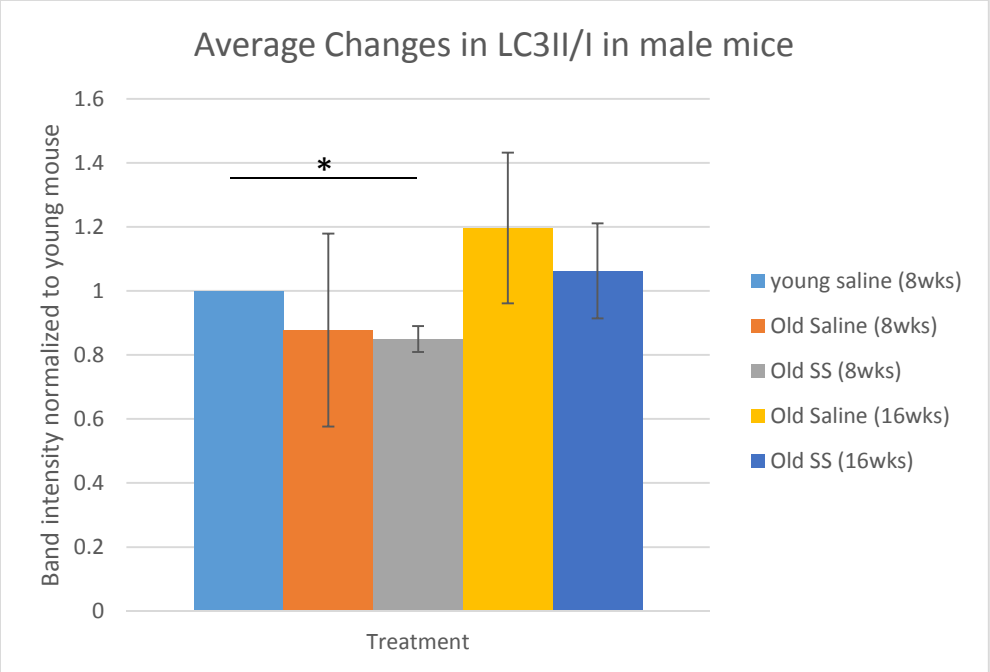


Figure 57: Average changes seen in LC3II/I protein levels in male mice. There is a significant decrease in the 8 week SS-31 treated group compared to the young saline treated group (*=p<0.05) (n=5). Error bars indicated standard deviation.

pAMPK/AMPK, another measure of autophagy and of proliferation, shows that there is no significant difference between any of the groups (figure 58). The 8 week SS-31 treated trend towards an increase in their pAMPK/AMPK expression level, but this lost 8 weeks later in the persistence mice, suggesting that SS-31 might play a role in this protein, but that it does not last for the 8 weeks following treatment.

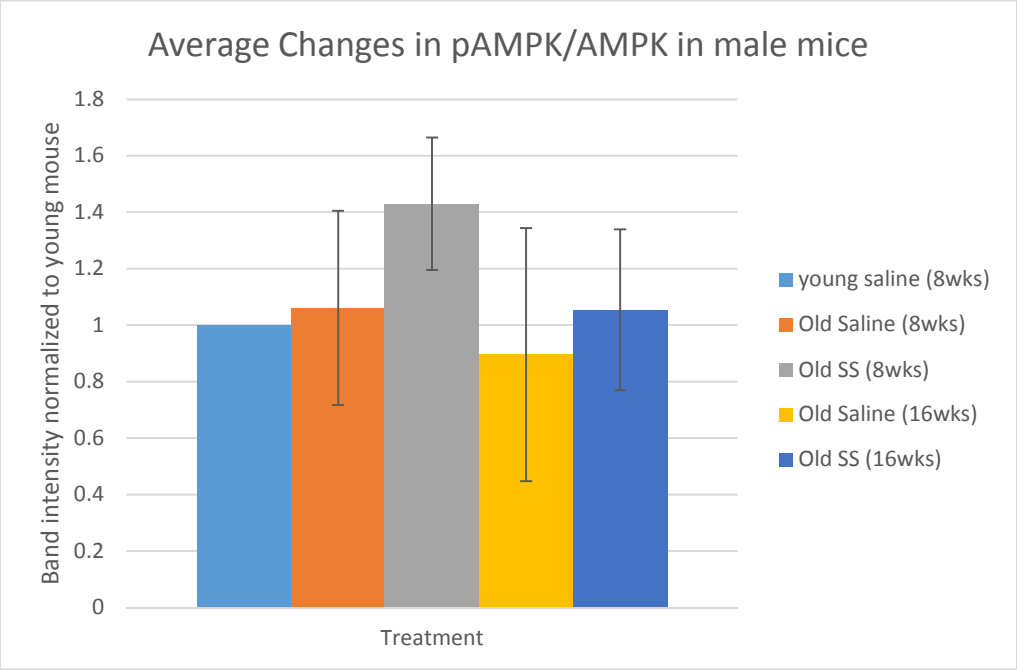


Figure 58: Male mice average changes in pAMPK/AMPK protein levels. There is no significant change between any of the groups (n=5). Error bars indicated standard deviation.

Investigating mitochondrial biogenesis by PGC1a shows only one significant change as seen in figure 59. The 28 month old SS-31 persistence treated group has a significant increase in PGC1a protein level compared to the young saline treated mouse. This suggests that these old mice are going through less efficient metabolic process to obtain their energy, despite having received SS-31. Additionally, SS-31 trends towards an increase at 8 weeks of treatment. While the 28 month old saline treated mouse has a decreased level compared to the young mouse or the 8 week SS-31 treated mouse. This suggests that the SS-31 peptide helps with mitochondrial biogenesis, primarily while the mice are still being treated with SS-31, but that some of this improvement is maintained eight weeks after drug removal. The trend towards a decrease in the old 16 week saline group suggests that mitochondrial biogenesis, but PGC1a protein levels, is decreased as mice age.

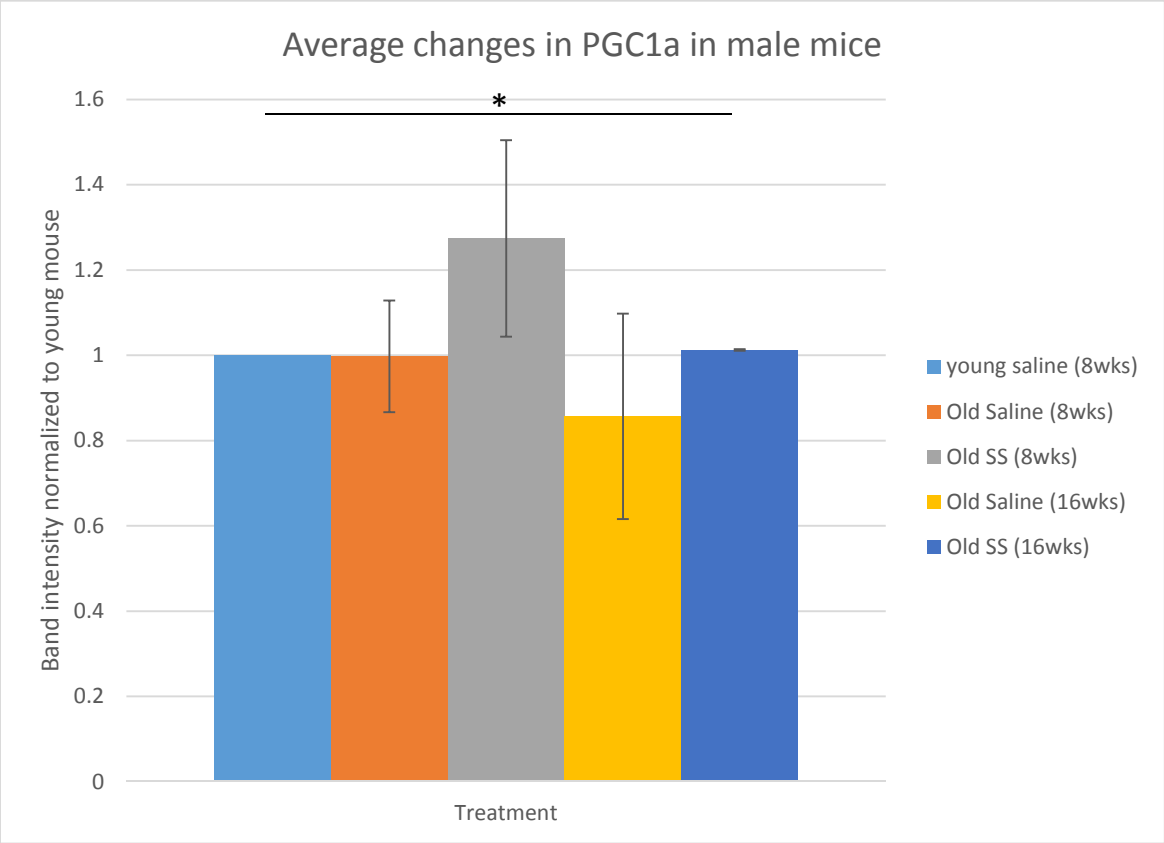


Figure 59: Average changes in PGC1a protein levels in male mice. There is a significant increase in the protein levels of the 16 week SS-31 persistent mice compared to the young saline control (*=p<0.05) (n=5). Error bars indicated standard deviation.

pAKT/AKT, a measure of cell proliferation, shows the most changes of all the protein levels we investigated in the SS-31 persistent mice which can be seen in figure 60. The SS-31 persistent mice had a significant decrease in their pAKT/AKT expression level compared to all the other treatment groups. The 28 month old saline treated mice also showed a significant decrease compared to the young and old saline treated mice. This again suggests that there might be a specific age change between the 26 month

old mice and the 28 month old mice compared to the treatment groups. However, due to the significant decrease seen in the SS-31 persistence group compared to its saline aged match control, there could be an effect due to the loss of SS-31. This conclusion would suggest that SS-31 helps change or stabilize the protein, that once removed has a larger negative effect compared to not giving the mice the drug at all. Future work would have to explore this more, and look into more specific aspect of the pathway.

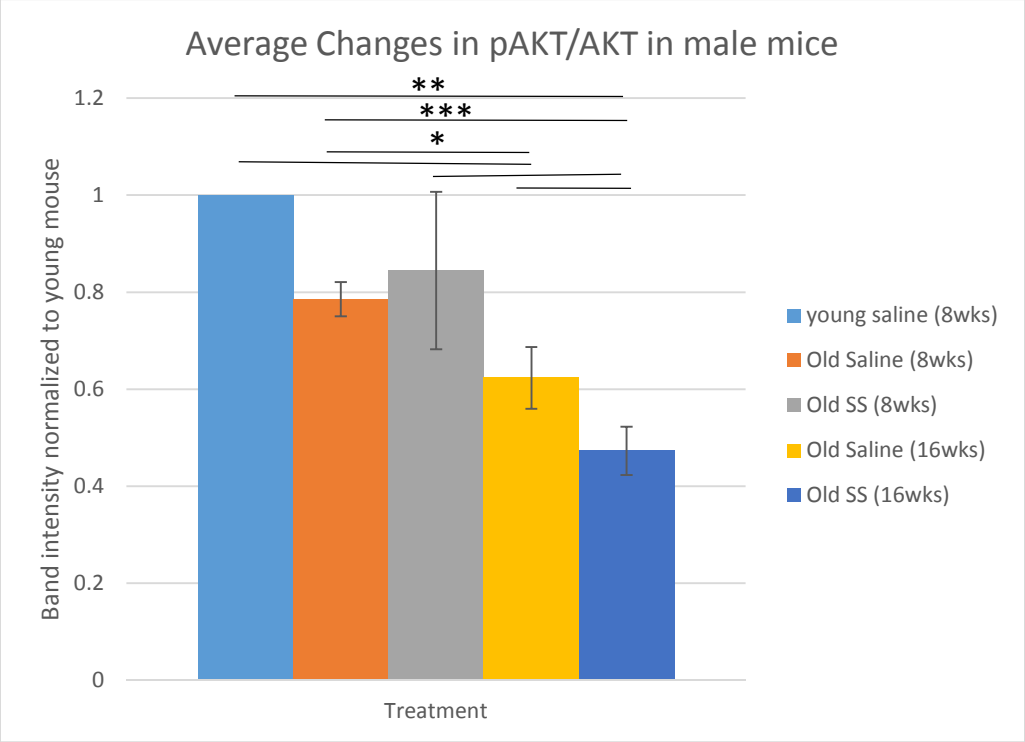


Figure 60: Average pAKT/AKT levels in male mice. There is a significant decrease in the protein levels of the SS-31 persistent mice compared to all other groups. There was also a significant decrease in the protein levels of the old 16 week saline treated group compared to the young saline and the 8 week saline treated mice (*=p<0.05, **=p<0.01, ***=<0.001) (n=5). Error bars indicated standard deviation.

While this protein level data is interesting and points to some of the same conclusions that were observed in the proteomics data clumping the age groups together, there is also a portion of the data that suggests that there is a relationship between the mice that received SS-31 and those that didn't. This data, while a good starting point, poses some challenges, including the small number of samples investigated. Each group only contains five animals, giving us insufficient power for many of the analysis, added to the large standard deviations seen amongst many of the groups. In fact, most of the significant changes occur in the groups with the smallest standard deviation. Future work would increase the biological samples within these groups to investigate the continuation of these conclusions. Additionally, all the results seen here are in male mice. As the proteomics pointed out, many of the changes seen are the same between the genders, but there are significant differences between them as well. Knowing this, it would be imperative to investigate the female mice to ensure that there are no gender differences and if there are, what conclusions could be drawn from them and the changes in cardiac aging by gender.

c. Electron microscopy

During the sacrifice of the mice in all cohorts, a small sliver of heart was removed from the mid-heart and stored in electron microscope fixative. These samples were then sent to our collaborator, Dr. Hazel Szeto, who took images of these samples. In figure 61, we see two magnifications of a young (4 mo old) heart sample. In the 8000X sample, the overall structure of the tissue shows the divided lines of mitochondria next to the myocardial fibers that use the energy to beat. The structure is highly organized. In the 80,000X sample, we see a closer image of the mitochondria. The cristae in the mitochondria all travel in the same direction as we would expect to see in a young healthy mouse.

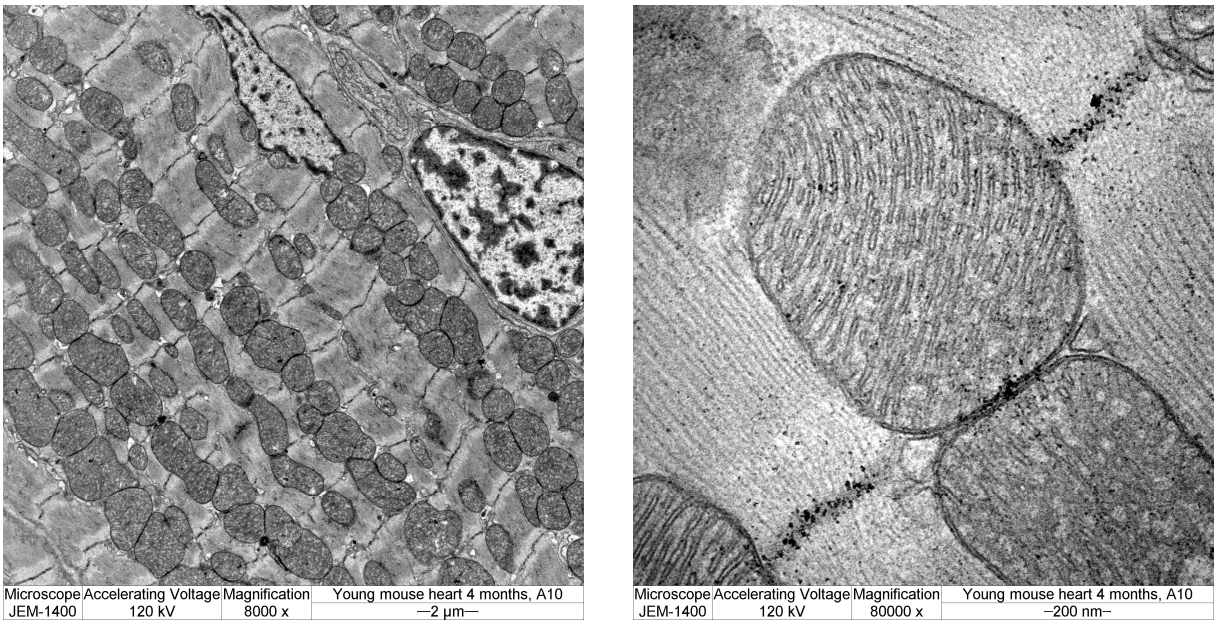
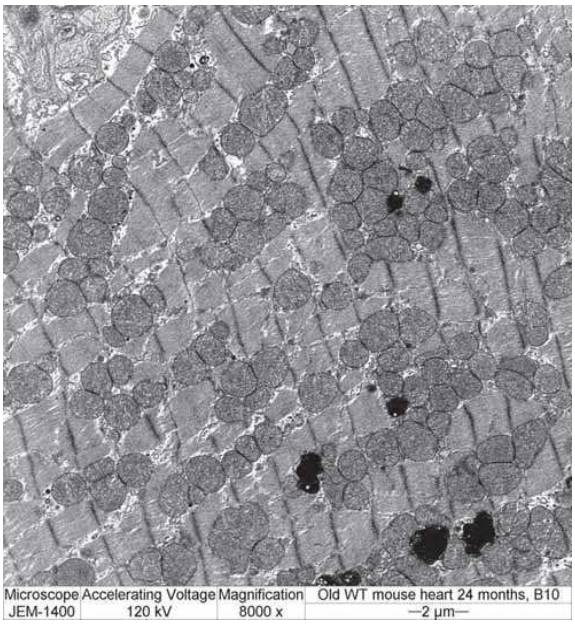


Figure 61: Electron microscope images of cardiac tissue from young (4mo old) mice. The mitochondria are organized in dividing lines of mitochondria and myofibrils. The cristae are organized at a 90 degree angle to the myofibrils, and the cristae are all aligned in the same direction.

In figure 62, we see a 24 month old mouse. The overall organization, as can been seen on the left hand side of the image, is not quite as structured as compared to the 4 month old mouse. There are more blobs of mitochondria, and not as many continuous lines of mitochondria compared to the young mouse. In the 80,000X magnification image, there is loss of cristae in the mitochondria, and they begin to curve in multiple directions. However, the myofibrils continue to look healthy.



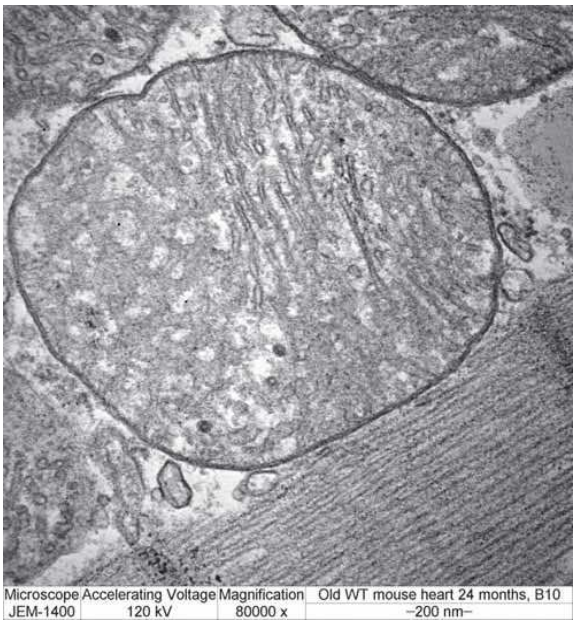
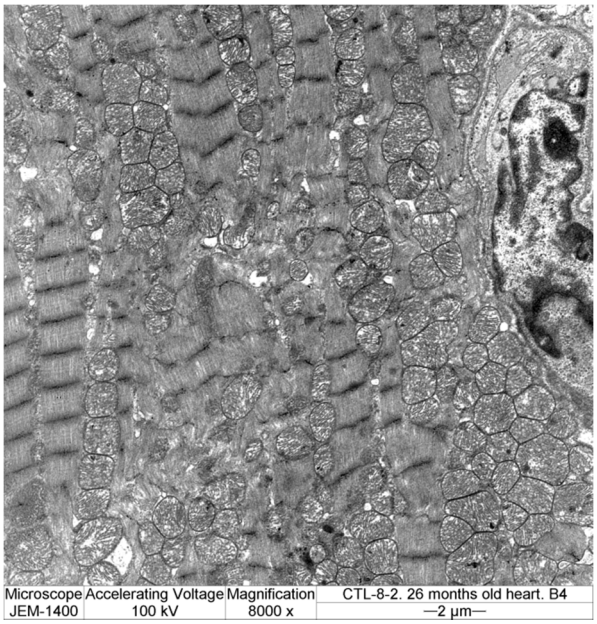


Figure 62: Electron microscope images of cardiac tissue from 24 month old C57/Bl6 mice. There are more disruptions in the organization of the mitochondrial lines. The cristae are at 90 degrees to the myofibrils.

At 26 months of age, with control treatment, the morphological structure of the mitochondria continues to deteriorate as can be seen in figure 63. Compared to earlier time points, either 4 month or 24 month mice, the mitochondria have lost the cristae within them, as well as the fact that the cristae, instead of being lined in the same direction, the cristae are disorganized within a single mitochondria, and the mitochondria nearby. The overall organization of the mitochondria, instead of having alternate lines of myofibrils and mitochondria, the mitochondria are banded together in larger conformations. Additionally, there are larger spaces between the myofibrils and their mitochondrial energy source.



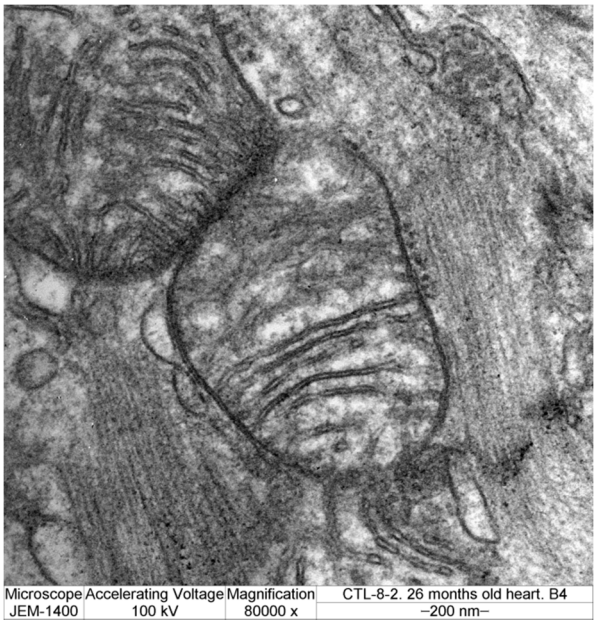


Figure 63: Electron microscope images of cardiac tissue of control mice at 26 months. In the 8000X image, there is a loss of organization of the mitochondria, with more blobs. At 80,000X there is loss of cristae and myofibril structure.

In contrast, as can be seen in figure 64, after 8 weeks of treatment with SS-31 there is an improvement in the morphology at the individual mitochondrial level and the overall cellular structure level. At the lower magnification, the myofibrils have a stronger definition, which is continued over at the 80,000X. The mitochondria at lower magnification follow a pattern similar to the young mouse, with the myofibrils and mitochondria arranged in alternate lines. This overall improvement mimics that of a mouse younger more than its 26 months of age.

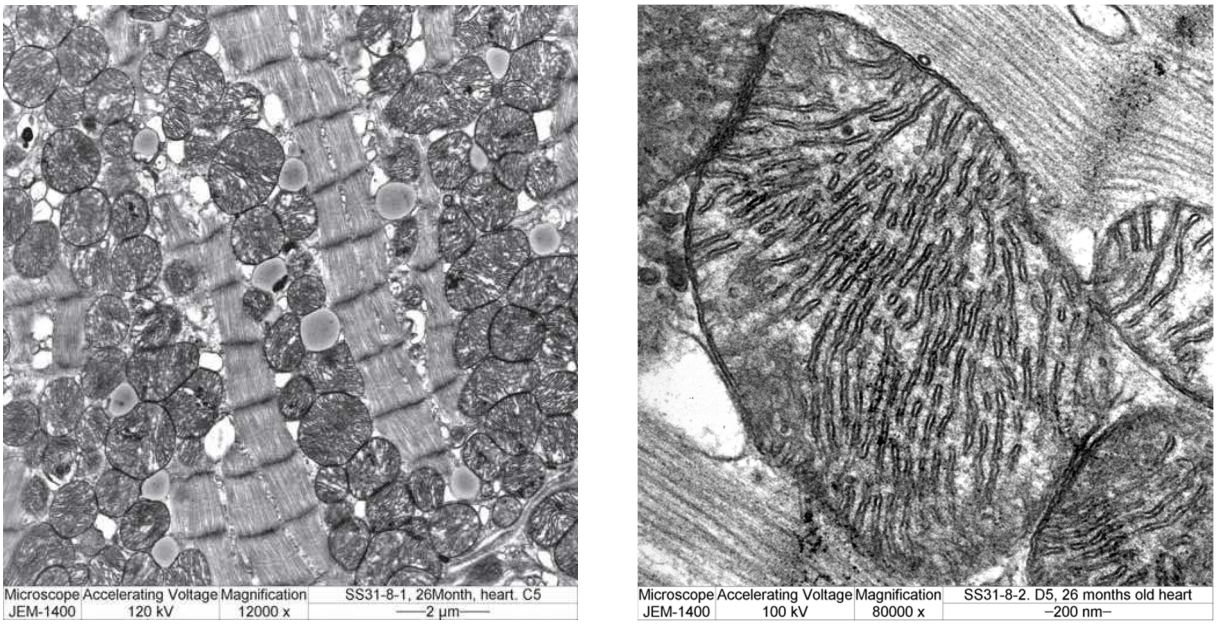
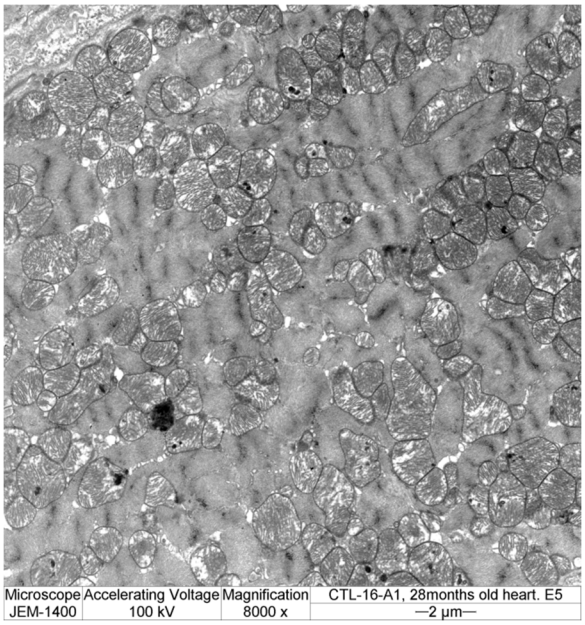


Figure 64: Electron microscope images of 26 month old SS-31 treated mice cardiac tissue. The 8000X image has organized myofibrils and mitochondria in alternating lines, with a few more blobs. At 80,000X, the mitochondria has well-formed cristae that are organized in the same direction, and at 90 degree angles to the myofibrils.

In figure 65, the electron microscope image of a 28 month old mouse, that was treated as a control for the first 8 weeks and then allowed to age normally, are shown. In the 8000X magnification, there is a large loss of myofibril structure, and the organization of the mitochondria is that of single mitochondria groups, not the organized, alternating stripes of myofibrils and mitochondria. At the 80,000X magnification, there is loss of the cristae in the majority of the mitochondria. Of the mitochondria that retain their cristae structure, the cristae are fragmented and beginning to lack alignment.



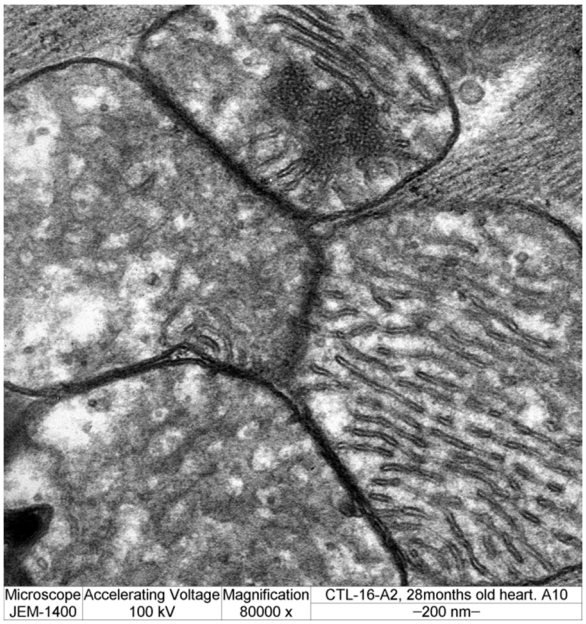
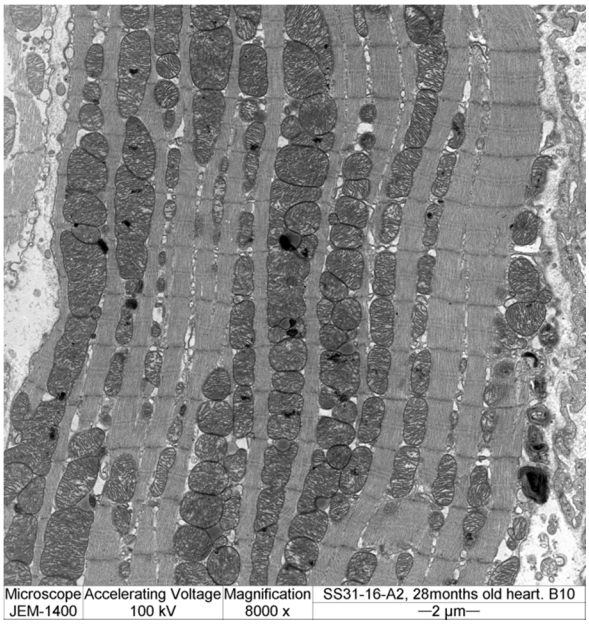


Figure 65: Electron microscope images of 28 month old control treated mouse cardiac tissue. There is loss of myofibril and mitochondrial structure. At the 80,000X image, a large portion of the mitochondria have lost the majority of the cristae structure.

In the SS-31 persistence treated mouse cardiac tissue, we see an improvement in morphology and structure as seem in figure 66. The arrangement of the myofibrils and mitochondria are orderly, with each one bordering each other, lining up the energy producer and the energy consumer. Additionally, the mitochondria have defined cristae that generally align in the same direction.



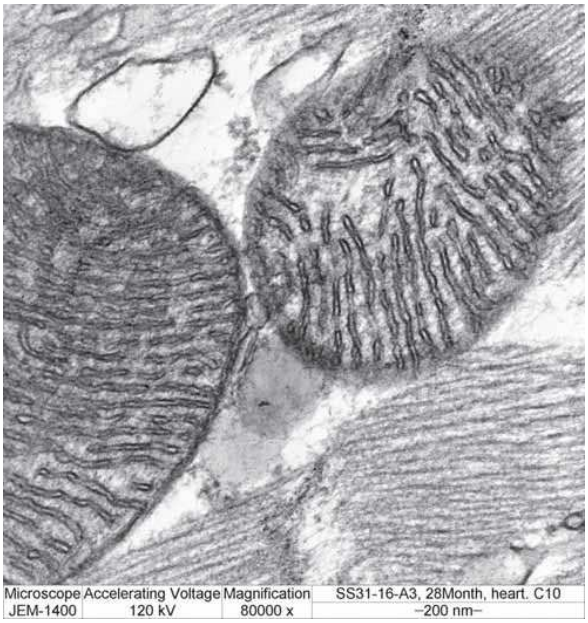


Figure 66: Electron microscope image of a 28 month old mouse that received 8 weeks of SS-31 before being allowed to age for an additional 8 weeks. In the 8000X image, the myofibrils and mitochondria form alternating stripes. In the 80,000X image, the cristae are aligned at 90 degree angles to the myofibrils, and generally are arranged in the same direction as each other.

Overall, the electron microscopy suggests that the SS-31 improves structural morphology of the mitochondria myofibril interaction and the cristae formation within individual mitochondria while being given to the mice, and that this effect persistence after the peptide is removed. This suggests that there would be improved cellular respiration and a decrease in reactive oxygen species from oxidative phosphorylation, by maintaining a strong intra-mitochondrial space for these processes to occur.

While these results are very promising, it should be noted, that images have currently only been captured for two mice from each group. Increasing the n for each group will be imperative, while measuring, quantitatively, the changes that are observed. This will include cristae number, orientation, and mitochondrial number.

VI. Conclusions

It does appear that SS-31 does have some persistent affects in the mice. Similar to the combined therapy data, we do not see the same changes in functional changes as have previously been observed in the lab (Chiao et al, unpublished). Additionally, there are no changes observed in hypertrophy at the time of sacrifice. The proteomic data that was obtained gives a very interesting interpretation of the total proteomic changes that are occurring in the heart at old age. The largest factor separating the various groups in the proteomics data was not any treatment group, but rather the age of the mice. This data suggests that there is a large proteomic shift in the heart for mice between the age of 26 and 28 months. This large shift is hiding many of the changes that might be seen between the treatments and the persistence of the SS-31 peptide. In future experiments, it would be wise to begin the treatment time point earlier, to help elucidate the differences seen due to age and the differences seen in treatment. The qPCR data obtained suggests that there is no effect of the SS-31, either with treatment or by persistence, in the markers of biogenesis, but there is an uptick in the amount of biogenesis transcript in older mice compared to young control mice. Similar to the combined treatment of these mice, the time points that we observe for the SS-31 persistence, might be too late for the biogenesis transcripts to still be up. Future studies should look at longer time points for biogenesis or other transcriptional markers that have a sustained rate change. Finally, the electron microscope images show a striking difference between the various treatment groups. The SS-31 peptide, not only appears to improve mitochondrial and myofibril morphology after 8 weeks of treatment, but that these morphological measures are sustained and continue to improve for at least another 8 weeks after treatment. Giving quantitative data to these observations is a top priority, as well as looking at possible proteins that might help with this improved morphology.

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