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Analyzing animal model morphometrics and the cardiac proteome in heart failure with preserved ejection fraction

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List of abbreviations

dKO – Double knockout group

ESC – European Society of Cardiology

HF – Heart failure

HFC – Heart failure cells

HFL – High fat diet with L-NAME group

HFL F – High fat diet with L-NAME group females

HFL M – High fat diet with L-NAME group males

HFpEF – Heart failure with preserved ejection fraction

HFrEF – Heart failure with reduced ejection fraction

L-NAME – N-nitro-l-arginine methyl ester

LVEF – Left ventricle ejection fraction

Obsc – Obscurin

Obsl1 – Obscurin like 1

SFD – Standard fat diet group

SFD F – Standard fat diet group females

SFD M – Standard fat diet group males

SR – Sarcoplasmic reticulum

Abstract

Introduction and Aims: Heart failure with preserved ejection fraction (HFpEF) is a diastolic dysfunction with unknown underlying mechanisms and etiology. A HFpEF mouse model was recently established, with mice on a high fat diet and water containing L-NAME which induces hypertension. A double knockout (dKO) of Obscurin/Obscurin-like1 (Obsl1) may also serve as a new model for HFpEF. The main purpose of this study is to investigate and compare changes to cardiac proteome between healthy lean mice, HFpEF mice and obscurin/Obsl1 dKO mice.

Methods: Cardiac samples from three male and three female HFpEF mice (HFL) were compared with three male and three female healthy lean mice (SFD). Depletion of actomyosin was made using dilution buffer and centrifugation, and before the samples were sent off to mass spectrometry for proteome-analysis, they were analyzed using SDS-page gel to see if the depletion had worked. Pre-existing lung-sections were deparaffinized and stained with Prussian Blue, pictures were then taken, and the HFC were counted. Student´s T-test and ANOVA with Post-Hoc Tukey test was used for statistical analysis between the groups on the proteomes, heart failure cells (HFC) and pre-existing data of the body weight, tibia lengths, water content in lungs and kidneys

Results: 128 significantly changed proteins found in the females, 56 in the males. HFL mice express significantly higher mean water content in lungs and kidneys. The HFL and dKO mice are significantly heavier than the SFD. No difference in tibia lengths. Significantly greater amount of HFC in dKO mice.

Conclusions: The results support the idea of HFpEF being a sex-specific disease that affects females more than males. Most proteins that are found in both the HFL and Obscurin/Obsl1 models are involved in the cardiac metabolism, mitochondrial function or in cardiac contractility, which supports the idea of the dKO model being a future HFpEF model.

Key words: HFpEF; proteome; Obscurin/Obsl1.

Introduction

Heart failure definition

Heart failure (HF) is defined by the European Society of Cardiology (ESC) as a clinical syndrome, which can be caused by structural and/or functional cardiac abnormalities, resulting in a reduced cardiac output and/or higher intracardiac pressures during stress or while resting. It is typical for heart failure patients to present additional symptoms such as dyspnea, fatigue and swelling in the ankles. Affected patients may also present with elevated jugular venous pressure and pulmonary crackles (1).

HF has been subclassified according to the ESC diagnostic criteria into 3 types depending on the measurement of left ventricular ejection fraction (LVEF) using echocardiography: HF with normal LVEF ≥50% is HF with preserved ejection fraction (HFpEF), HF with LVEF <40% is HF with reduced ejection fraction (HFrEF), HF with an LVEF between 40-49% is considered a ”grey area” and is therefore HF with a midrange ejection fraction (HFmrEF). HFpEF is a diastolic HF, which means that the filling ability of blood into the heart chambers is disturbed, while systolic HF, which is seen in HFrEF patients, is when the heart cannot pump out enough blood to the system. An additional criteria for HFpEF is elevated natriuretic peptides (1).

HFpEF is a more challenging syndrome to diagnose because a diverse population group and limited amount of patient heart tissue biopsies makes it difficult to search for potential molecular pathways that are involved in the etiology of the disease, and there is therefore also difficulty in the development of drugs. In addition, the development and analysis of experimental HFpEF models is difficult, as patients have a range of associated comorbidities such as renal disease, hypertension and diabetes mellitus (2).

Epidemiology

HFpEF is the leading form of HF in the US, with half of all 6.5 million HF patients suffering from this syndrome. The number of HF patients has been stable throughout the years, but there has been an increasing incidence of HFpEF while HFrEF has had a decreasing incidence. The incidence of HFpEF also increases with age (3), and many studies show that the typical HFpEF patient is an older woman with hypertension (4). The female to male ratio

is 2:1 (5). Other risk factors include diabetes, obesity, coronary artery disease/ischemia and chronic kidney disease (3).

HFpEF unfortunately has a high morbidity and mortality rate: about 35% of those who have been hospitalized for this syndrome have a 5-year survival. Those with comorbidities, which is common, have an even higher risk of death (6).

Etiology

The etiology is still somewhat unknown and most likely differs from HFrEF. Compared to HFrEF patients, valvular heart disease, hypertension and atrial fibrillation is seen more often in HFpEF patients, while myocardial infarction and left bundle branch block is seen less in HFpEF patients. HFpEF patients also have a significantly higher incidence of elevated blood pressure, which is a main cause of stiffness in arteries and higher afterload of the left ventricle, lower resting heart rate and lower plasma-potassium levels (4).

Although the pathophysiology is still barely understood, mostly because of the multi-faceted nature of comorbidities being common in these patients, there are several mechanisms that have been suggested to play a role in the etiology of the syndrome. One suggested cause of HFpEF is systemic low-grade inflammation that has been mediated through tumor necrosis factor alpha (TNF-alpha) and transforming growth factor beta 1 (TGF-beta 1), but the amount of fibrosis in the myocardium does not correlate with the severity of the deterioration of diastolic function in HFpEF (4). Other suggested mechanisms include depression of β-adrenergic signaling, reduced recoil of elastic elements and oxidative stress targeting calcium-handling proteins (2).

Actin, myosin and titin form three essential filament types in the sarcomere, which is the contractile apparatus in all cross-striated muscle cells. Repeated sarcomeres in series form myofibrils. Myofibrils are surrounded by the sarcoplasmic reticulum (SR) membrane system (7). Calcium is released from the SR to initiate muscle contraction. Calcium release is triggered by an electrical stimulus that binds to ryanodine receptors which will release SR-bound calcium. Calcium ions will be pumped back into the SR by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) when contraction ends. The SR therefore works as a special type of membrane compartment that is responsible in storing the cells calcium (8). The proteins obscurin (Obsc) and obscurin-like 1 (Obsl1) are localised to the M-band, which is a

structure in the middle of the sarcomere, through interactions with myomesin and titin. For the giant protein obscurin it has been shown that it connects the sarcomere to the SR. Even though obscurin is mainly expressed in striated muscle tissue, there is also expression in non-muscle tissues, and it is known that Obsl1 shows a ubiquitous expression pattern (9). Preliminary studies at the UC San Diego Department of Cardiology have shown that cardiac specific double knockout mice for obscurin and Obsl1 (dKO) recapitulate many of the features found in HFpEF patients, including profound diastolic dysfunction with normal systolic function, abnormal left ventricular relaxation and premature death (Figure 1). Single obscurin or Obsl1 knockouts do not display HFpEF-characteristics or shortened life expectancy compared to the wildtype controls.

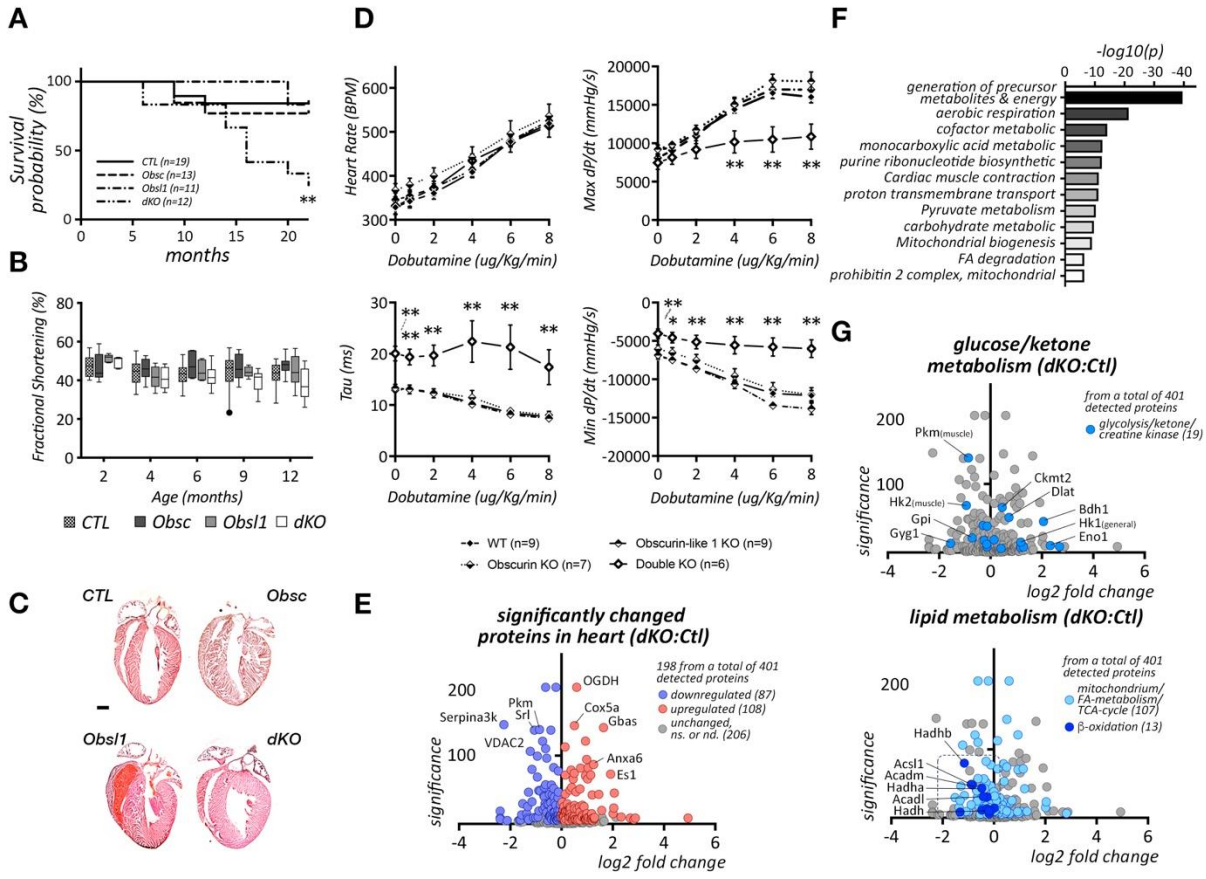

Figure 1. Unpublished results from Lange and others Obscurin/Obsl1 dKO HFpEF mouse model. A. Cardiac specific obscurin/Obsl1 double knockout mice (dKO) display shortened life-expectancy compared to wildtype controls (CTL), cardiac specific Obsl1 knockouts or obscurin (Obsc) knockouts. B-C. Left ventricular fractional shortening as measured by echocardiography (B) and gross cardiac morphology (C) are unchanged between dKO, Obsl1, Obsc and control mice. D. Obscurin/Obsl1 dKO mice show diastolic dysfunction (Min dP/dT) and cardiac relaxation problem (Tau) at baseline (0µg/Kg/min Dobutamine), and lack of cardiac reserve under stress (by dobutamine). * $p<0.05$, ** $p<0.01$ vs. CTL. E. Proteome analysis between control (CTL) and dKO hearts revealed 198 significantly deregulated proteins. F-G. Enrichment analysis (F) revealed characteristic changes to glucose and ketone metabolism as well as beta-oxidation (G), which are

present in the most significantly changed pathways: generation of precursor metabolites and energy, aerobic respiration and cofactor metabolic. Unpublished data.

However, it is unknown if obscurin/Obsl1 dKO mice may serve as a new model for HFpEF, and which molecular features can be found in other mouse models for this syndrome or in patients.

L-NAME and high fat diet

Recently, a novel two-hit HFpEF mouse model was established, triggered by metabolic and hypertensive stress that were on a high fat diet and drinking water containing N-nitro-l-arginine methyl ester (L-NAME) (10). The study showed that this animal model recapitulated many of the clinical characteristics of HFpEF. Using longitudinal echocardiographic, preserved LVEF was observed in these mice and measurements by noninvasive Doppler and invasive analyses showed an increased left ventricular filling pressure. These mice also had a lung weight increase which indicates that there is pulmonary congestion and that a preclinical state of HF has evolved. Other findings were cardiac and cardiomyocyte hypertrophy, cardiac fibrosis and myocardiac capillary rarefaction. Eventually, after 15 weeks with this diet, exercise intolerance was observed compared to the control group even though there was no sign of skeletal muscle function impairment, therefore indicative of heart problems. Also, stress in the endoplasmic reticulum and chronic adiposal inflammation has been seen in mice that have been fed with a high fat diet for 16 weeks (11).

Proteomes and proteomics

Proteome is all the set of proteins expressed in a cell, tissue or organism encoded by a genome. Proteomics is a way of studying and characterizing the proteome, which can be done by using technologies such as mass spectrometry (12). Mass spectrometry is usually the preferred technology due to its ability of handling the proteome´s complexities (13).

Heart failure cells and pulmonary edema

Heart failure cells (HFC), also called siderophages, are macrophages located in the alveoli of heart failure patients. They contain hemosiderin is because they have phagocytized erythrocytes that have leaked from the congested lung capillaries due to HF and then degraded hemoglobin (14).

Pulmonary edema is when the lungs fill with liquid between the interstitial and alveolar spaces, which results in the body struggling to get enough oxygen and shortness of breath occurs. It can be seen in HFpEF patients due to it being caused by an elevated cardiac filling pressure (15).

Gap-of-knowledge

There is a gap-of-knowledge for precise molecular pathomechanisms at play in HFpEF etiology, caused by a caused by a diverse patient population, range of associated comorbidities, the dearth in patient biopsy samples and available pre-clinical models to study the disease.

Aims

The purpose of this study is to get a better understanding of the mechanisms at play in HFpEF etiology. We compared two mouse models of the disease on a proteomic level, by investigating clinical changes of the heart and lungs using pre-existing data, and by histological staining of lung sections. All comparisons and analyses are done between healthy lean mice, HFpEF mice and obscurin/Obsl1 dKO mice.

Materials and Methods

Animals

This project analyzes an established model for diet and hypertension induced HFpEF (10). Prior to the start of the project, 10 male and 10 female mice had undergone diet induced obesity by having being fed high fat diet (60 kcal% fat) from 3 until 16 weeks of age, when metabolic syndrome and adipose inflammation were present (11). These mice had in addition to high fat diet been treated with L-NAME (0.5 g/L in drinking water). This group is called the High Fat diet with L-NAME group (HFL). The control group involves 10 male and 10 female mice who had been fed with normal chow (10 kcal% fat) and untreated drinking water instead. This group is called the Standard Fat Diet group (SFD). Cardiac samples were isolated and processed for protein analysis. An inbred mouse strain (C57/BL6) was used for this analysis, and the mice should therefore be genetically similar. In addition, all mice were littermates, with always 5 mice housed together. The extraction of cardiac samples from the mice had already been done prior to the start of the project.

Analyzing the cardiac proteome

Extraction of cardiac proteins

Biobanked cardiac samples from a total of 12 mice were used (see Table 1). 3 male mice who were on a standard fat diet (SFD M), 3 female mice who were on a standard fat diet (SFD F), 3 male mice who were on a high fat diet with L-NAME (HFL M) and 3 female mice who were on a high fat diet with L-NAME (HFL F).

Table 1. Group distribution with their samples´ identities.

SFD M	SFD F	HFL M	HFL F
SFD 7	SFD 16	HFL 8	HFL 16
SFD 8	SFD 17	HFL 9	HFL 17
SFD 9	SFD 18	HFL 10	HFL 18

SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F - Female.

Each frozen cardiac sample was first placed in a tube and mixed with 0.5 ml of ice cold lysis buffer and homogenized using a polytron blade. 6.5 ml of lysis buffer was made by mixing 1.95 ml of 1 M KCL, 0.391 ml of 0.5 M pipes, 0.1635 ml of 20% NP40, 0.26 ml of protease inhibitor, 0.26 ml of Phosphate inhibitor and 3.4755 ml of H2O. The lysis buffer helped to disrupt the cells which helped release the intracellular components. Each tube was then sonicated and centrifugated for 10 minutes in 15000 revolutions per minute at 4 °C, which was done to isolate soluble proteins from insoluble cell-debris. The supernatant was then pipetted into 12 new tubes, while the pellets were discarded.

Protein quantification

Bio Rad Protein Assay (2 mg/ml) and Optical Density 595 (OD 595) were then used to measure the concentration of proteins in the solutions for subsequent mass-spectrometry analysis. Using a Bio Rad Protein Assay and water, 7 different BSA Protein concentrations were made; 0 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml and 2 mg/ml. The BSA solutions were then used in OD 595, which measures absorptions and determines the intensity of proteins. With the results from the OD 595, a linear curve with a constant value of 0.8149 was made. The samples were then placed in the OD 595. Using the constant value and the results of the samples from the OD 595, the concentration of protein in the samples could be determined with the help of the following equation.

Y/0.8149 = X

Y = OD 595

X = Concentration

The 12 samples were then put into 4 different tubes. The 3 SFD M samples in one (SFD 7,8 and 9), SFD F samples in one (SFD 16, 17 and 18), HFL M samples in one (HFL 8, 9 and 10) and HFL F samples in one (HFL 15, 16 and 17). To have the same concentration of protein of each sample in the new tubes, different amount of volume was taken from each solution. SFD 7, 8 and 9 had approximately the same concentration of protein and 0.5 ml of each sample was therefore put into the tube. SFD 16, 17 and 18 had different concentrations, with SFD 17 having the lowest concentration of 0.594 and 0.5 ml of it was therefore put in the tube. While SFD 16 had a concentration of 0.647, (0.594/0.647) x 0.5 = 0.46 ml of it was therefore put into the tube. This was done with the other groups as well (see Table 2.).

Table 2. The OD595 results for each sample. With their calculated concentrations a volume to use was determined.

ID	OD595	Concentration (mg/ml)	Volume into combined tube (ml)
SFD 7	0.876	1.075	0.5
SFD 8	0.815	1.000	0.5
SFD 9	0.885	1.086	0.5
SFD 16	0.527	0.647	0.46
SFD 17	0.460	0.594	0.5
SFD 18	0.650	0.797	0.37
HFL 8	1.196	1.467	0.42
HFL 9	1.236	1.517	0.40
HFL 10	0.998	1.225	0.5
HFL 15	0.781	0.958	0.41
HFL 16	0.639	0.784	0.5
HFL 17	1.010	1.239	0.32

SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, OD-595 – Optical Density 595.

20 ul of each tube was then pipetted into 4 new tubes and blended with 20 ul sample buffer. This is the All group (A group), which contain all the proteins, including myosin and actin.

This solution was only centrifugated once before to extract fragile proteins and other subcellular organelles that are not relevant in the analysis. This was later used for SDS-PAGE gel with Coomassie blue staining.

Depletion of actomyosin and preparation of proteins for subsequent mass-spectrometry analysis

The remaining of the 4 sample groups were then diluted with dilution buffer. 26 ml of dilution buffer was made by blending 1 phosphatase inhibitor tablet, 1 protease inhibitor tablet, 52 ul 1mM DTT, 650 ul 0.5% NP-40 and 25.3 ml H2O. Each group was diluted in a ratio of 1:4. This was done to decrease the amount of salt, which precipitates actomyosin into pellets. The solutions were then centrifugated for 10 minutes in 15000 revolutions per minute at 4 °C. The supernatants were then pipetted into 4 new tubes. 20 ul of the supernatant is then mixed with 20 ul of the blue sample buffer, this is the Mass-Spectrometry groups (MS groups) and they contain all the proteins except for myosin and actin. They were later sent to the Core facility for Mass-Spectrometry. The pellets of each group were mixed with 100 ul of the blue sample buffer (P groups). These should contain the majority of actomyosin and interacting proteins since actin and myosin become insoluble with the decreased salt amount and they pellet. In the end, there were 12 tubes; 4 containing all of the proteins (A group), 4 containing all of the proteins except for actomyosin (MS group), 4 containing only myosin and actin (P group).

Analysis of actomyosin depletion by SDS-Page

To test if the actomyosin depletion worked, the samples were loaded up in wells in an electrophoresis SDS-PAGE gel with Coomassie blue staining (see Table 3). The molecular weight marker was used for determining the approximate size of a protein run on the electrophoresis gel. All the wells needed to be filled, and since there were 15 wells but only 12 samples, well 2 and 15 were loaded up with Sample Buffer. Since the proteins in the pellets were highly enriched and had to be dissolved in sample buffer, less were pipetted on to the gel compared to the input or the supernatant fraction for SFD (or HFL). If the same amount were to have been pipetted, then the gel would have overloaded. This experiment was not specifically designed to measure or compare protein amounts, but to see if the pellet fraction contains myosin and actin, which had to be depleted from the protein lysate to allow for detection of less abundant proteins. Relative volumes were kept between samples though (e.g. 2ul SFD M Pellet, 2ul SFD F Pellet, etc) to allow for comparison within each fraction (ie. Compare all pellet fractions with each other).

Table 3. The loading in each well of the gel.

Loading	Sample
1	2 ul Molecular Weight Marker
2	5 ul Sample Buffer
3	5 ul SFD M All
4	5 ul SFD F All
5	5 ul HFL M All
6	5 ul HFL F All
7	2 ul SFD M Pellet
8	2 ul SFD F Pellet
9	2 ul HFL M Pellet
10	2 ul HFL F Pellet
11	20 ul SFD M MS
12	20 ul SFD F MS
13	20 ul HFL M MS
14	20 ul HFL F MS
15	5 ul Sample Buffer

SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F – Female, MS – Mass spectrometry.

The gel shows that there is myosin and actin in the A samples, mostly only myosin and actin in the P samples and in the MS samples the rest of the proteins yet still some myosin left (see Figure 2). Using the program Fiji, the intensity of each myosin band is measured (Table 4), which shows the percentage of myosin depletion in the MS-samples compared to the A samples. The MS samples are sent to the Core Facility for mass-spectrometry.

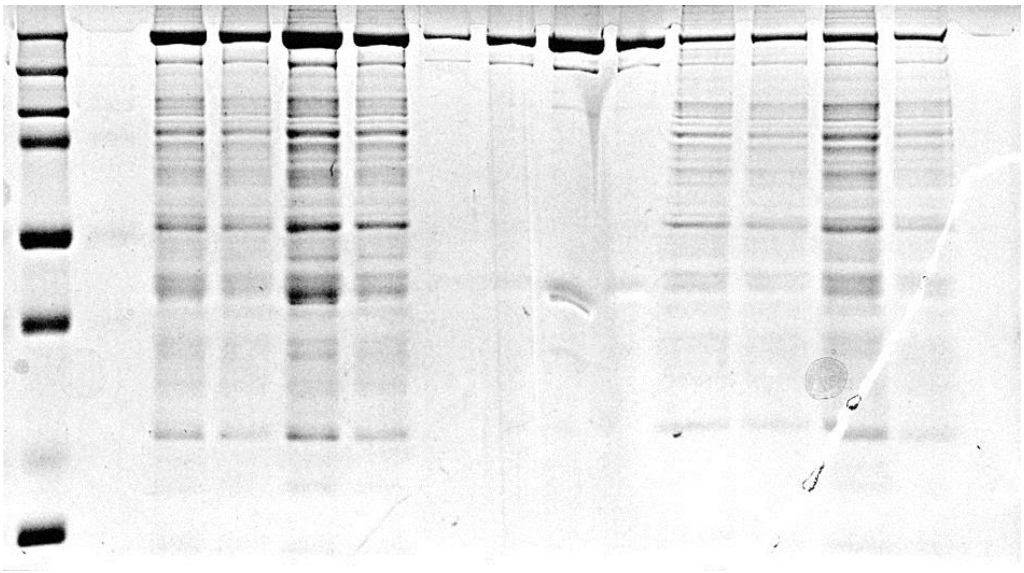

Figure 2. SDS-PAGE gel. From left to right: Molecular Weight Marker, Blue Sample Buffer, SFD M All (n=3), SFD F All (n=3), HFL M All (n=3), HFL F All (n=3), SFD M Pellet (n=3), SFD F Pellet (n=3), HFL M Pellet (n=3), HFL F Pellet (n=3), SFD M MS (n=3), SFD F MS (n=3), HFL M MS (n=3), HFL F MS (n=3) and Blue Sample Buffer. Strongly stained bands on the top of the gel are myosin (~250 kDa). *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F – Female, MS – Mass spectrometry, n – number.*

Table 4. The intensity measurements/raw values of the myosin bands in the SDS PAGE gel in each sample, and the percentage of myosin in the Pellet and MS groups compared to the All groups. SFD M Pellet (n=3) and SFD M MS (n=3) are compared with SFD M All (n=3), SFD F Pellet (n=3) and SFD F MS (n=3) are compared with SFD F All (n=3), HFL M Pellet (n=3) and HFL M MS (n=3) are compared with HFL M All (n=3), HFL F Pellet (n=3) and HFL F MS (n=3) are compared with HFL F All (n=3).

Samples	Raw values	Percentage of mysin compared to the All groups.
SFD M All	**11857.03**	1
SFD F All	**8164.79**	1
HFL M All	**15170.52**	1
HFL F All	**10318.21**	1
SFD M Pellet	**2827.36**	0.238454
SFD F Pellet	**6426.33**	0.787078
HFL M Pellet	**12726.30**	0.838883
HFL F Pellet	**7935.69**	0.769096
SFD M MS	**4735.36**	0.399371
SFD F MS	**5109.18**	0.625758
HFL M MS	**6649.18**	0.438296
HFL F MS	**4268.01**	0.413639

SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F – Female, MS – Mass spectrometry, n – number.

Figure 3 presents a summarization of the experiment.

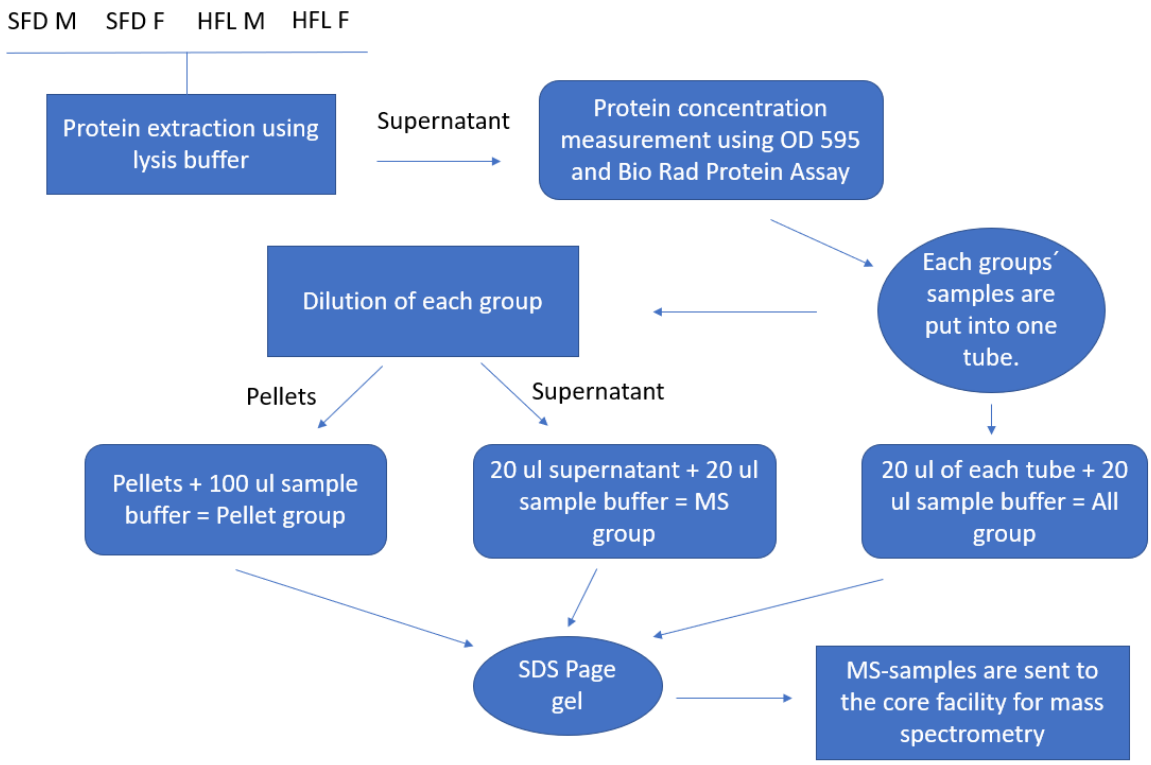

Figure 3. Summarization of the experiment. N=3 in each group. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F – Female, MS – Mass spectrometry, OD 595 – Optical Density 595, n – number.*

Analyzing the cardiac proteome

The results from the core facility indicate that thousands of differentially expressed proteins between SFD and HFL are identified. We performed three analyses, comparing all differentially expressed proteins between SFD and HFL males, SFD and HFL females and a combined SFD-HFL comparison that disregards sex as a biological variable. The significance cut-off is based on prior experience using similar set of samples and was set at 15, decreasing the number of proteins to analyze. The manufacturer of the software used to analyze the proteome data suggests a cut off of 20: "A significance score threshold of 20 is recommended, which equals to a significance testing p value of 0.01." (16). Proteins are presented as gene symbols based on their significance and log2 fold change (FC). The more positive a number of FC of a gene is, the more expressed it is in the HFL group compared to the SFD group, meanwhile if it is negative, it is less expressed in the HFL group. A FC value can measure between +8 and -8, where +8 means expression only in HFL and -8 means expression only in SFD.

Pre-existing data

To see if there was a significant difference between the healthy lean mice and HFpEF mice regarding the water content in the lungs and kidneys, body weight and tibia lengths, statistical analysis was performed on the pre-existing data. In addition, Obscurin/Obsl1 dKO mice was compared with the SFD and HFL groups regarding the water content percentage in lungs, body weight and tibia lengths, even though there was only data from 3 mice. The sample identities for the dKO group are OL2421, OL2471 and OL2472.

Table 5. The sample identities of the SFD, HFL and dKO groups and their data. The data for water content percentage in kidneys of OL mice is unknown.

Sex	ID	Tibia (mm)	Body weight (g)	Water content% Lung	Water content% Kidney
Male	SFD 1	18.55	31	0.168	0.256
	SFD 2	18.23	31	0.136	0.254
	SFD 3	18.19	31	0.146	0.254
	SFD 4	18.41	31	0.219	0.251
	SFD 5	17.69	31	0.175	0.245
	SFD 6	18.53	29	0.198	0.245
	SFD 7	18.38	33	0.156	0.23
	SFD 8	18.07	29	0.226	0.247
	SFD 9	17.85	31	0.171	0.235
	SFD 10	18.13	31	0.191	0.231
Female	SFD 11	18.11	23	0.211	0.256
	SFD 12	18.31	25	0.156	0.242
	SFD 13	17.79	23	0.162	0.242
	SFD 14	17.69	23	0.148	0.249
	SFD 15	18.27	23	0.201	0.248
	SFD 16	19.66	24	0.213	0.241
	SFD 17	17.96	22	0.147	0.254
	SFD 18	17.54	23	0.188	0.248
	SFD 19	17.41	24		0.239
	SFD 20	18.09	25	0.14	0.249

Male	HFL 1	0	0		
	HFL 2	17.8	47		0.283
	HFL 3	17.91	55	0.231	0.25
	HFL 4	11.1	50	0.246	0.272
	HFL 5	18.02	46	0.312	0.283
	HFL 6	17.67	42	0.234	0.309
	HFL 7	18.42	47	0.255	0.25
	HFL 8	18.38	46	0.211	0.303
	HFL 9	18.11	43	0.23	0.237
	HFL 10	18.02	46	0.201	0.286
Female	HFL 11	18.23	42	0.267	0.297
	HFL 12	18.07	33	0.21	0.281
	HFL 13	18.6	36	0.206	0.274
	HFL 14	18.46	36		
	HFL 15	18.09	29	0.191	0.239
	HFL 16	17.74	41	0.312	0.279
	HFL 17	18.57	50	0.22	0.301
	HFL 18	17.91	32	0.238	0.253
	HFL 19	0	31		
	HFL 20	17.8	36	0.168	0.263
Male	OL2471	19.08	54.2	0.34	
	OL2472	17.99	35.8	0.20	
Female	OL2421	18.18	29.5	0.15	

SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, OL – the IDs of double knockout mice.

HFL 1, HFL 14 and HFL 19 were excluded from the analysis because of sudden death before the endpoint of the treatment period was reached. Therefore, no measurements were recorded for these animals. SFD 19 and HFL 2 were excluded as well, because the measurements of the water content % of the lungs were too high to be correct, and therefore they most likely had been wrongly measured.

Heart failure cells

Pre-existing PFA fixed histological paraffin sections of lungs needed staining before being observed with an optical microscope. 4 samples from the SFD group, 4 from the HFL group and 3 samples from the dKO group were used (see Table 6).

Table 6. Group distribution with their samples´ identities. SFD 1, SFD 5, HFL 3, HFL 5, OL2471 and OL2472 are males. SFD 17, SFD 18, HFL 11, HFL 18 and OL2421 are females. The HFL and SFD mice were 5 months of age when the data was taken. The dKO mice were 9 months of age when the data was taken.

SFD	**HFL**	**dKO**
SFD 1	HFL 3	OL2421
SFD 5	HFL 5	OL2471
SFD 17	HFL 11	OL2472
SFD 18	HFL 18	

SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, dKO - Double knockout group, OL – the identities of double knockout mice.

For them to be stained, a deparaffinization was first needed to extract the paraffin because the staining has a hydrophilic buffer and paraffin is hydrophobic. The slides were put in xylene 2 x 3 min, 100% ethanol 2 x 3 min, 95% ethanol 3 min, 70% ethanol 3 min, 50% ethanol 3 min, then running cold tap water to rinse and they were in tap water after the deparaffinization until they got stained, because if they had gotten dry there would be a non-specific antibody binding and therefore also a high background staining. Since HFC were the cells to identify, Prussian Blue Staining was chosen so that the iron in these cells could be detected. The staining was done by mixing equal parts of 20% hydrochloric acid and 10% potassium ferrocyanide, after which the slides were put in the solution for 20 minutes. Thereafter they were washed in distilled water, with 3 changes, and then dehydrated with 95% ethanol and 2 changes of 100% ethanol. After that, clearing with 2 changes xylene was done, 3 minutes for each change. Lastly, a coverslip was mounted with Permount embedding medium.

To search for HFC in the lung sample slides of mice, an optical microscope was used. Iron in siderophages/HFC should be bright blue when stained with Prussian Blue, therefore allowing for the identification of these cells. 5 pictures were taken of each sample-slide, with every picture being of a different location on the slide. The HFC in the pictures were then counted and normalized to imaged lung area, and statistical analysis was performed of the results to see if there was a significant difference between the groups.

Statistical methods

The program IBM SPSS was used to perform the statistical analysis of the results of the pre-existing data and the HFC comparisons. The choice of statistical method were: Student's T-test when comparing the means of two groups and ANOVA with post-hoc Tukey HSD Test when comparing more than 2 groups. This was done on the pre-existing data and the HFC comparisons.

To get the significance for the proteome data, an ANOVA analysis was performed using the PEAKS program. The PEAKS manual cites the following manuscript for their statistical analysis: "On the comparison of several mean values: an alternative approach" Biometrika. 1951, 38(3/4): 330-336 (16).

Student´s contribution

All of the forementioned methods were performed by me under the supervision of Dr. Lange, who has taught me how to correctly execute experiments by supervising me during each step.

Ethics

There are ethical considerations present in this study, since it includes experiments done on vertebrate animals (mice). However, all involved procedures on the mice have been approved by the Institutional Animal Care and Use Committee (protocol No. S13009). Because of the close similarities between the body of a mouse and the human body (17), and that the use of mice in this case could result in the treatment of an untreatable disease makes the research ethically and morally justified. Without the potential benefits of this research, HFpEF patients would most likely have to wait longer for a treatment, which would result in these patients having to keep on suffering from the disease and having a shortened life-expectancy.

Results

Comparison between the cardiac proteomes in SFD and HFL males

Table 7 presents a total of 56 differentially expressed proteins between SFD M and HFL M that had a significance value ≥15 in order from the highest to the lowest in significance. Figure 4 presents a volcano plot to easier highlight protein significance according to their FC.

Protein Pdk4 (pyruvate dehydrogenase kinase, isozyme 4) had the highest significance value (significance 95.15) and a positive log2 FC (FC 1.29), meaning it is upregulated in the HFL group. Ighg2c (immunoglobulin heavy constant gamma 2C) has a high significance (significance 73.31) and is downregulated in the HFL group (FC -1.18). Mrps18b (mitochondrial ribosomal protein S18B), Hdgfrp2 (hepatoma-derived growth factor-related protein 2), Klhl6 (kelch-like family member 6), Ewsr1 (EWS RNA-binding protein 1) and Cecr6 (cat eye syndrome chromosome region, candidate 6) are only expressed in the HFL group (FC 8), while Setx (senataxin) is only expressed in the SFD group (FC -8).

Using Metascape, the biological pathways of the significantly changed proteins are identified (see Figure 5), with the most significant being cardiac muscle contraction.

Table 7. Significantly differentially expressed proteins between SFD M and HFL M.

Males		
Gene name	**Log2 FC**	**Significance**
Pdk4	1.29278175	95.15
Ighg2c	-1.1844246	73.31
Pon1	1.38404981	54.37
Plin5	1.02147973	44.3
Igkappa	-0.9159357	41.91
Mrpl27	1.9068906	41.56
Acot2	0.79077204	41.3
Mrps18b	8	38.9
Hdgfrp2	8	38.42
Rhog	0.80735492	38.41
Setx	-8	37.29
Klhl6	8	36.82
Ewsr1	8	36.68
Cecr6	8	36.58
Myh7	0.75702325	32.82
Lyz1	0.87970577	31.71
Lyz2	0.87970577	31.71
Ech1	0.67807191	31.55
Hmgcs2	0.90303827	28.2
Cav1	-0.7858752	27.54
Hspb7	0.84799691	26.06
Cox4i1	-0.5994621	25.53
Mtatp6	0.83187724	25.51
Decr1	0.59454855	25.28
Fbp2	0.64154603	23.69
Cat	0.55581616	22.87
Scp2	0.61353165	22.49

Bdh1	-0.5353317	21.88
Hsd17b11	0.68706069	21.76
Igtp	0.79908731	21.36
Ttr	0.52606881	20.88
Vdac2	-0.5353317	20.84
Mtco2	-0.5563933	20.59
Vwa8	0.51601515	19.89
Cox6c	-0.5353317	19.15
Tnni3	-0.4941091	19.13
Cox6b1	-0.5145732	19.11
Vdac3	-0.4941091	18.93
Vdac3-ps1	-0.4941091	18.93
Krt78	-0.8624965	18.52
Hspa1a	-0.7131189	18.24
Hspa1b	-0.7131189	18.24
Ephx2	0.48542683	17.96
Aqp1	-0.5353317	17.77
Krt2	-0.5145732	17.64
Myl3	-0.4739312	17.02
Ucp3	0.65992456	16.8
Acadvl	0.45417589	16.44
Ighm	-0.4739312	16.03
Myh6	-0.4540316	15.95
Acox1	0.52606881	15.64
Myl2	-0.4344028	15.5
Oxct1	-0.4344028	15.45
Acsf2	0.48542683	15.38
Hadha	0.43295941	15.2
Mipep	0.61353165	15.09

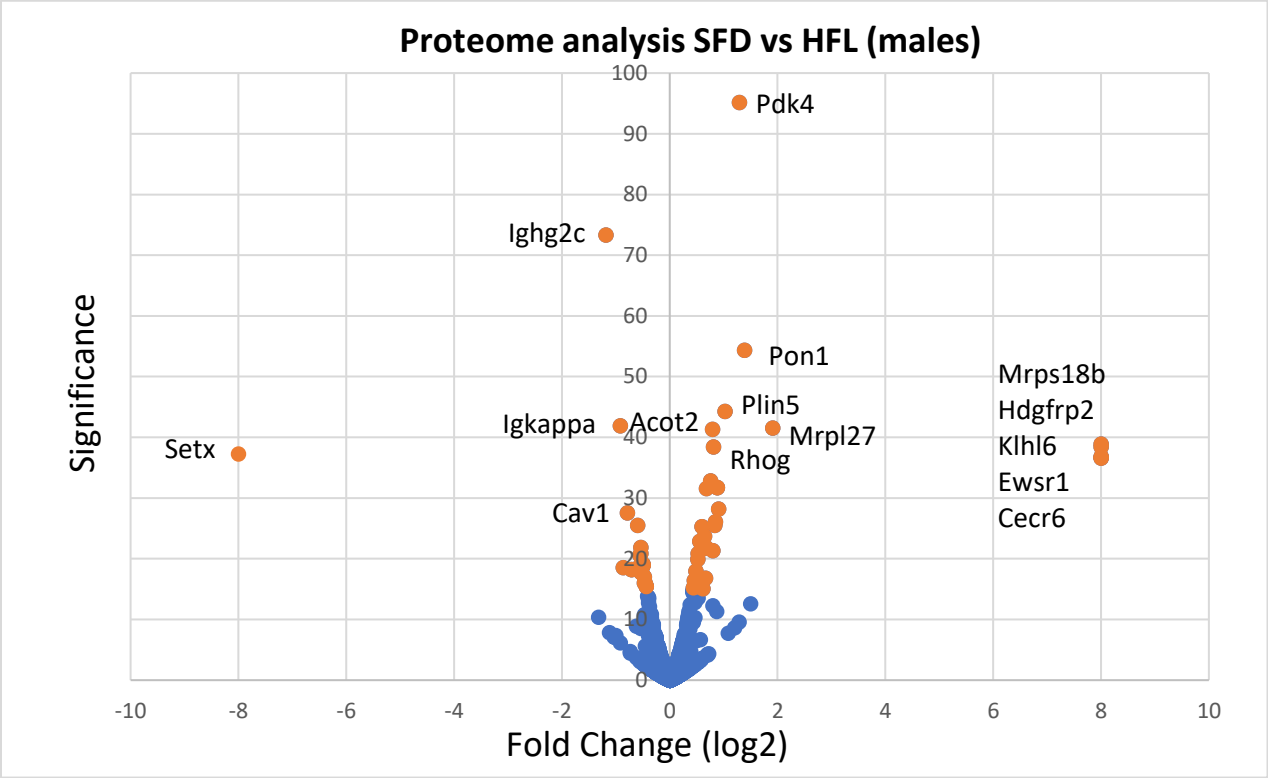

Figure 4. Volcano plot illustrating the significance according to the fold change of the proteins in the male SFD (n=3) vs HFL (n=3) proteome. The orange dots present the proteins with a significance value ≥ 15. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, n – number.*

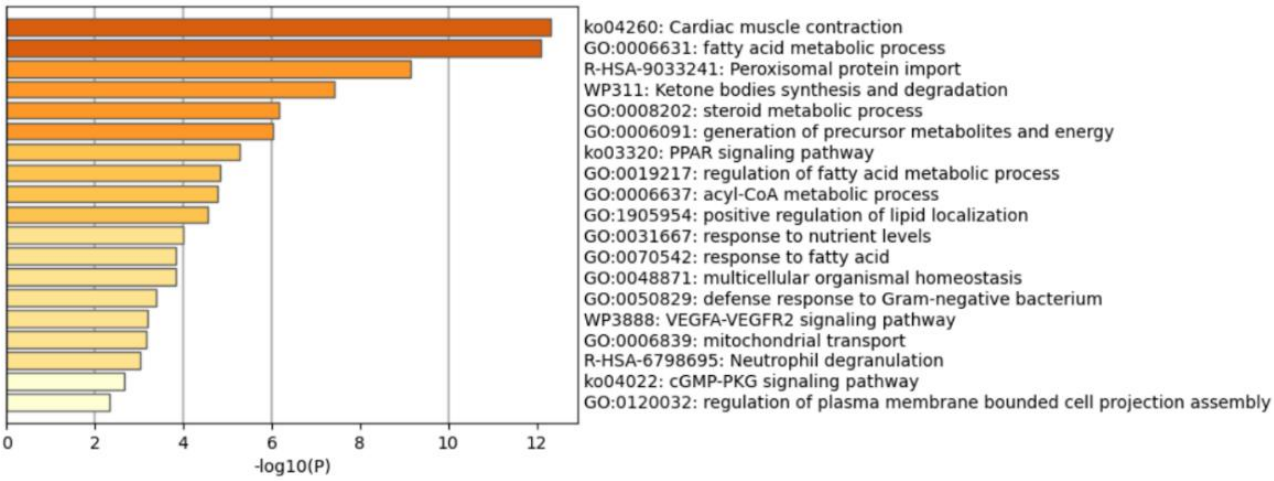

Figure 5. The biological processes that are highly deregulated in HFL M (n=3), in order from highest significance to lowest. *HFL - High fat diet with L-NAME group, M – Male, n – number.*

Comparison between the cardiac proteomes in SFD and HFL females

Table 8 and Figure 6 display a total of 128 proteins in the female SFD vs HFL proteome analysis. Mtco1 (cytochrome c oxidase subunit I), Anxa1 (annexin A1), and the lysozymes

Lyz1 and Lyz2 have the highest significance (significance 200) and are downregulated in the HFL group (log2 FC -2.74, -2.06, -3.84, -3.84). Axin1, Trim41 (tripartite motif containing 41) and Cecr6 are only expressed in HFL (log2 FC 8), while Hdgfrp2, Ppp2r5e (protein phosphatase 2, regulatory subunit B', epsilon isoform), Numa1 (nuclear mitotic apparatus protein 1), Prrc2a (proline-rich coiled-coil 2A) and Klhl6 are only expressed in the SFD group (FC -8).

Figure 7 presents biological pathways of the significantly changed proteins, with the most significant being signaling by Rho GTPases.

Table 8. Significantly differentially expressed proteins between SFD F and HFL F.

Females		
Gene name	**Log2 FC**	**Significance**
Mtco1	-2.7369656	200
Anxa1	-2.0588937	200
Lyz1	-3.8365013	200
Lyz2	-3.8365013	200
Hist1h3b	-2	143.11
Hist1h3a	-2	143.11
Coro1a	-2.1202942	139.05
Gsn	-1.4739312	114.45
Arhgdib	-1.5994621	110.35
Hist1h4a	-1.5563933	103.53
Rhog	-1.5145732	103.38
Mgst3	-2.2515388	102.98
Pdk4	1.32192809	87.18
Ndufa11	-1.4739312	75.94
Mpo	-1.5145732	72.65
Lcp1	-1.4739312	72.07
Mtnd4	-1.6438562	63.9
Cycs	1.03562391	63.82
Mtatp6	-1.5145732	62.77
Actb	-1	62.41
Actg1	-1	62.41
Mtnd1	-1.8889687	62.35
Acot2	0.97819563	60.37
Cox8b	-2.5563933	60.11
Myh9	-1.1844246	58.08
Myl4	-1.1520031	50.86
Mb	-1.1844246	48.88
S100a1	-3.8365013	43.2
Tkt	-0.8889687	41.78
C1qbp	-1.2515388	41.36

Myl6	-1.1202942	41.05
Krt10	-0.7858752	40.41
Myl7	-1	36.27
Hp	-1.1202942	35.69
Cfl1	-0.9714308	34.36
Krt2	-0.7858752	33.67
S100a6	-1.0892673	33.42
Nisch	-1.9434165	33.25
S100a4	-1.8365013	31.96
Pfn1	-0.7612131	31.31
Mp68	2.25701062	31.06
Decr1	0.66902677	30.86
Ech1	0.66902677	30.67
Ptbp1	-1.9434165	29.21
Cybb	-1.6896599	28.69
Hist1h1b	-0.9434165	28.49
Flna	-0.6214884	27.76
Hspb2	-0.6438562	27.49
Gbe1	-0.7369656	27.21
Cap1	-0.6665763	27.16
Acaa2	0.60407132	25.65
Krt5	-0.7369656	25.64
Mylpf	-1.6896599	25.44
Rpl32	1.49569516	25.31
Fabp5	-0.6896599	25.15
Tpm3-rs7	-0.6665763	24.67
Slc25a42	0.80735492	24.47
Apoa4	0.72246602	24.23
Ppp6r1	-1.5994621	24
Rps18-ps3	1.70043972	23.59
Gm10260	1.70043972	23.59
Rps18	1.70043972	23.59
Hsdl2	0.56559718	23.17
Mecp2	0.70487196	22.97
Setx	-4.6438562	22.24
Hist3h2bb	-3.0588937	22.19
Hist2h2be	-3.0588937	22.19
Mmp9	-4.0588937	22.17
Scp2	0.67807191	22.08
Hdgfrp2	-8	21.91
Ppp2r5e	-8	21.9
Numa1	-8	21.52
Prrc2a	-8	21.43
Hspa1a	-0.9714308	21.39
Hspa1b	-0.9714308	21.39
Axin1	8	21.38
Gm17669	0.64154603	21.36
Rpl29	0.64154603	21.36

Trim41	8	21.34
Klhl6	-8	21.29
Cecr6	8	21.24
Hmgb2	-1.3219281	21.06
Vwa8	0.52606881	20.68
Hmgcs2	0.83187724	20.55
Arpc1b	-0.8889687	20.35
Mup13	-2.8365013	20.31
Gps1	-0.7858752	20.13
Vim	-0.6214884	19.99
Krt78	-1.1844246	19.56
Hadha	0.50589093	19.46
Dcn	-0.5353317	19.37
Lta4h	-0.5994621	19.35
Ndufa9	-0.577767	19.19
Pbp2	-2.7369656	18.84
Tagln	-0.5994621	18.6
Cpt2	0.48542683	18.35
Acsf2	0.62293035	18.11
Acadvl	0.47508488	17.85
Agpat3	0.60407132	17.84
Psma4	-0.5563933	17.72
Mpi	-0.5994621	17.51
Actn1	-0.5563933	17.49
Arid2	-2.5563933	17.33
Plin5	0.64154603	17.27
Ola1	-0.5145732	17.22
Xpo7	-1.3219281	16.78
Gnb2	-0.577767	16.73
Hsd17b11	0.65076456	16.67
Mipep	-0.7131189	16.59
Mvp	-0.5994621	15.99
Acadl	0.44360665	15.95
Txnl1	-0.7369656	15.94
Cdh13	-0.6438562	15.85
Glud1	-0.4344028	15.77
Myl12b	-1.0588937	15.65
Myl12a	-1.0588937	15.65
Nudt9	-1.1844246	15.55
Alb	-0.4344028	15.5
Arpc4	-1.1520031	15.42
Gmps	-0.6896599	15.39
Hibadh	0.43295941	15.37
Ahsg	-0.4941091	15.35
C4b	-0.6896599	15.33
Rpl23a	0.67807191	15.25
Rpl23a-ps3	0.67807191	15.25
Akr1b10	-0.4344028	15.22

Apoh	-0.5563933	15.18
Mars2	-0.6438562	15.15

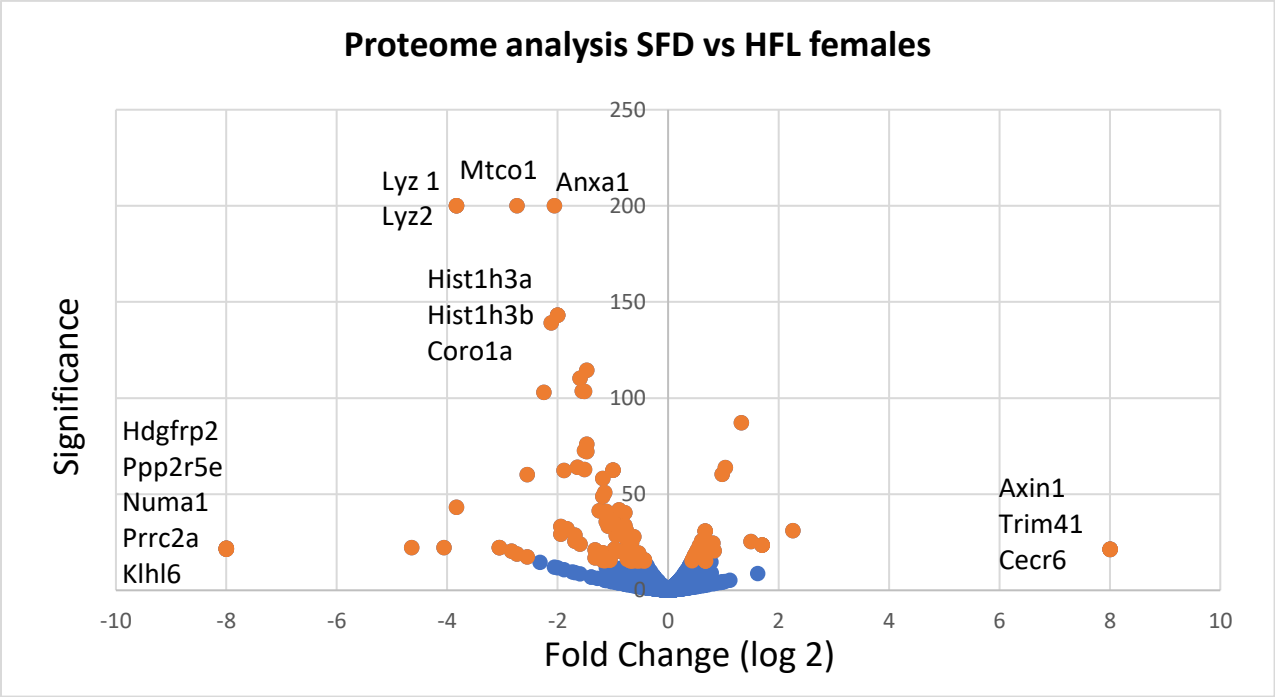

Figure 6. Volcano plot illustrating the significance according to the fold change of the proteins in the female SFD (n=3) vs HFL (n=3) proteome. The orange dots present the proteins with a significance value ≥ 15. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, n – number.*

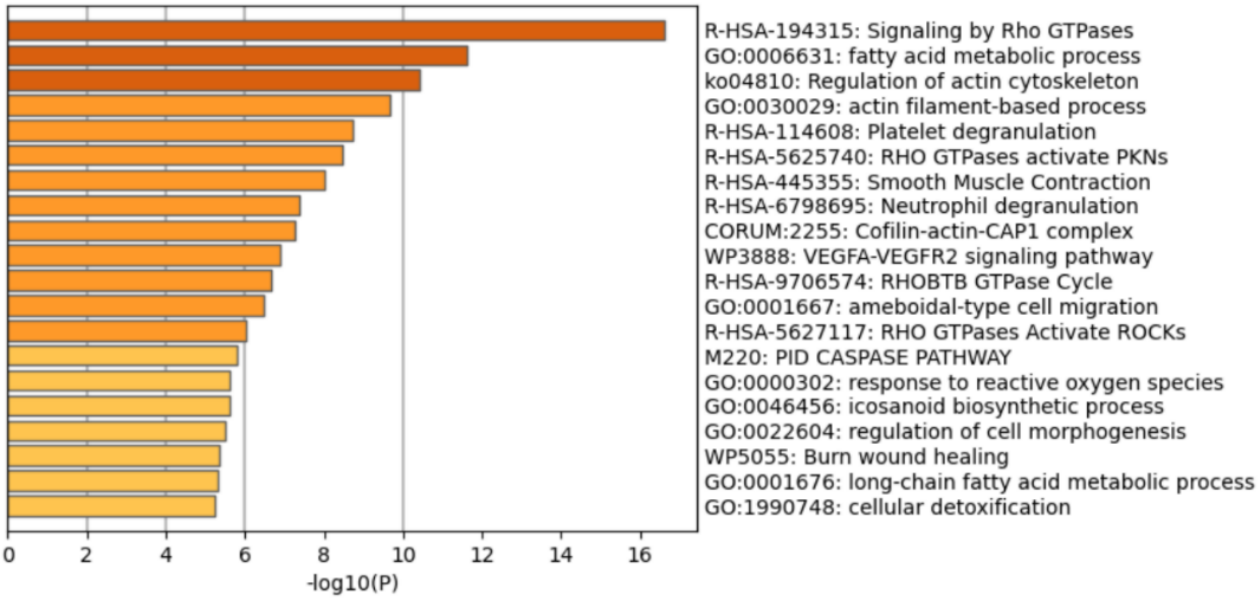

Figure 7. The biological processes that are highly deregulated in HFL F (n=3). *HFL - High fat diet with L-NAME group, F – Female, n – number.*

Comparison between the cardiac proteomes in SFD and HFL mice, regardless of sex as a biological variable

Table 9 and Figure 8 presents a total of 29 differentially expressed proteins between SFD and HFL mice, when excluding sex as a biological variable. Lysozymes Lyz 1 and Lyz 2 have the highest significance (significance 81.45) out of all the male and female proteins. Trim 41 and Cecr 6 also have a high significance value (significance 45.67) and are only expressed in the HFL group (FC 8).

Figure 9 presents biological pathways of the significantly changed proteins.

.

Table 9. Significantly differentially expressed proteins between SFD and HFL

Males and females combined		
Gene name	**Log2 FC**	**Significance**
Lyz1	-2.6438562	81.45
Lyz2	-2.6438562	81.45
S100a1	-2.9434165	58.18
Mtco1	-2.1202942	56.06
Setx	-4.6438562	47.2
Mmp9	-3.3219281	46.96
Trim41	8	45.56
Cecr6	8	45.56
Cox8b	-1.7858752	34.11
Mgst3	-1.6896599	29.86
Coro1a	-1.5563933	29.63
Anxa1	-1.4739312	28.5
Hist3h2bb	-2.2515388	28.1
Hist2h2be	-2.2515388	28.1
Mup13	-2.1844246	26.7
Pdk4	1.30451104	25.27
Hist1h3b	-1.358454	24.59
Hist1h3a	-1.358454	24.59
Pbp2	-1.9434165	22.62
Hist1h4a	-1.2175914	21.58
Nisch	-1.358454	20.63
Arid2	-1.8365013	20.38
Prrc2a	-1.7858752	19.44
S100a4	-1.2863042	19.32
Arhgdib	-1.0892673	17.9
Ptbp1	-1.2863042	17.61
Sec24d	-1.6438562	16.76
Cybb	-1.1520031	16.04
Krt78	-1.0588937	15.97

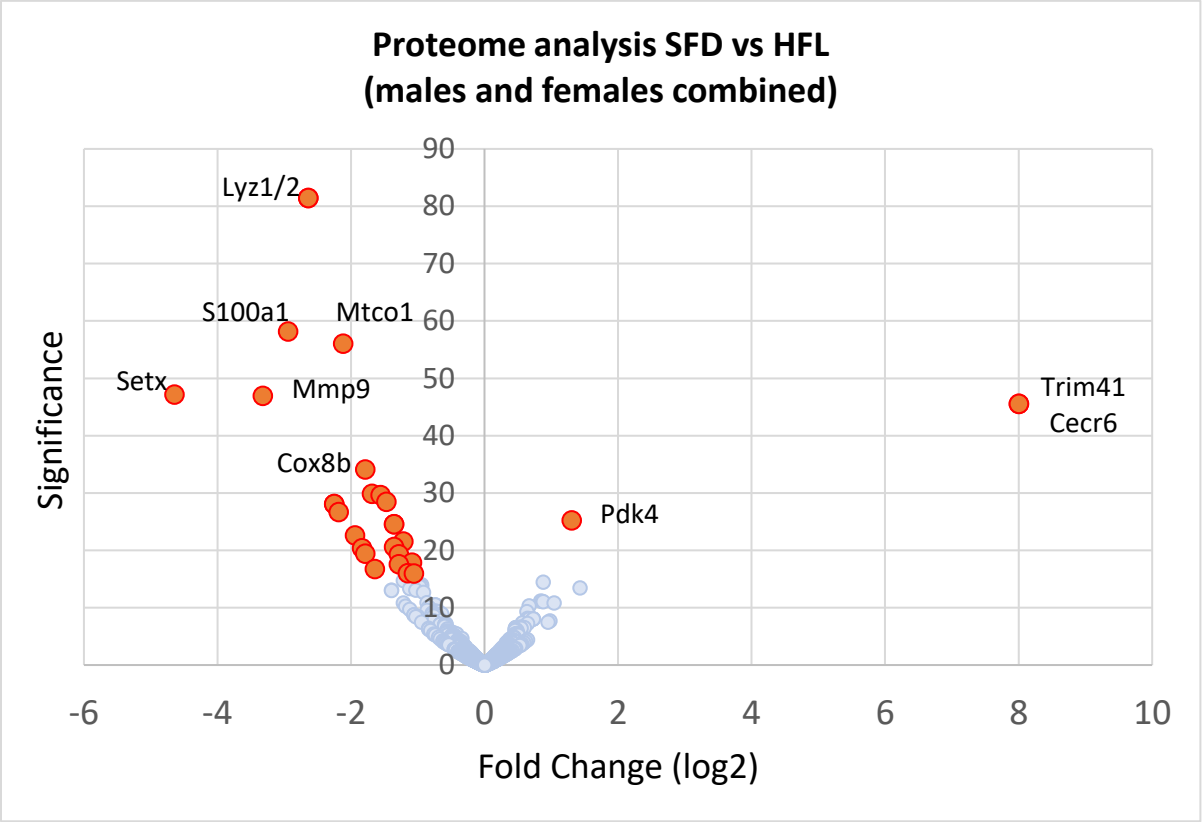

Figure 8. Volcano plot illustrating the significance according to the fold change of the proteins in a combined male (n=6) and female (n=6) SFD vs HFL proteomes. The orange dots present the proteins with a significance value ≥ 15. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, n -number.*

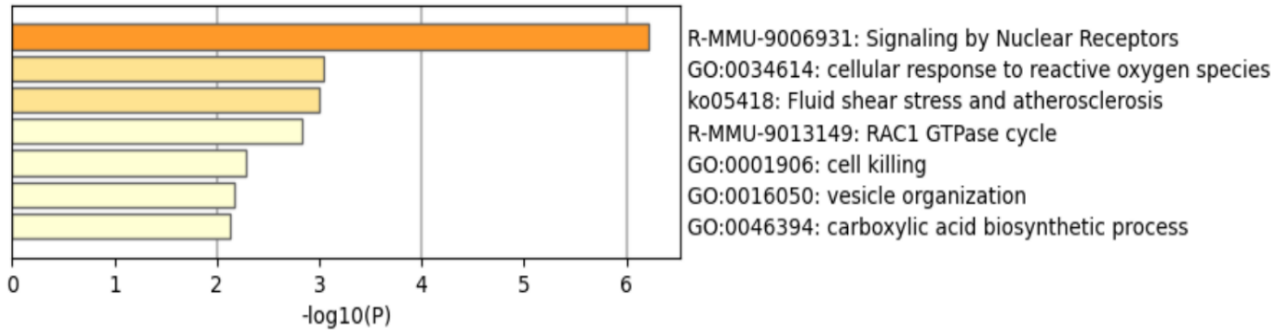

Figure 9. The biological processes that are highly deregulated in HFL (n=6) regardless of sex as a biological variable. *HFL - High fat diet with L-NAME group, n – number.*

Morphometric comparisons SFD/HFL mice with obscurin/Obsl1 dKO mice

Lung and kidney organ congestion

Figure 10 displays a bar graph which compares the mean water content percentage in the lungs between SFD M, HFL M, SFD F and HFL F. Using SPSS, Independent-Samples T-test presented a significantly greater percentage of water content in HFL mice than SFD mice when comparing both groups with each other, with a 5.68% mean difference of water content ($p = 0.000023$). A comparison between SFD M and HFL M had a 6.14 % difference ($p = 0.001$), and SFD F and HFL F had a 5.25 % difference ($p = 0.012$). ANOVA with post-hoc Tukey HSD test showed no significance when comparing SFD M with SFD F or HFL M with HFL F.

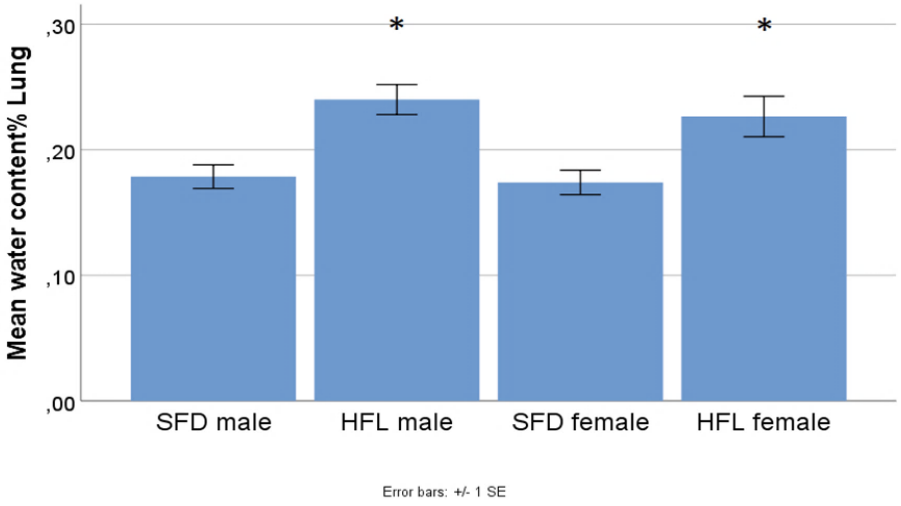

Figure 10. Comparison of mean water content percentage in the lungs between SFD M (n=10), HFL M (n=8), SFD F (n=9) and HFL F (n=8). The asterisks indicate statistical significance. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F – Female, n – number.*

Using ANOVA, the mean of water content percentage in the lungs of obscurin/Obsl1 dKO was not significantly higher in comparison to SFD ($p = 0.122$; see figure 11). However, this result was influenced by the low sample size (n=3) and high standard error for obscurin/Obsl1 dKO mice, indicating that a higher sample size is needed for a robust statistical analysis.

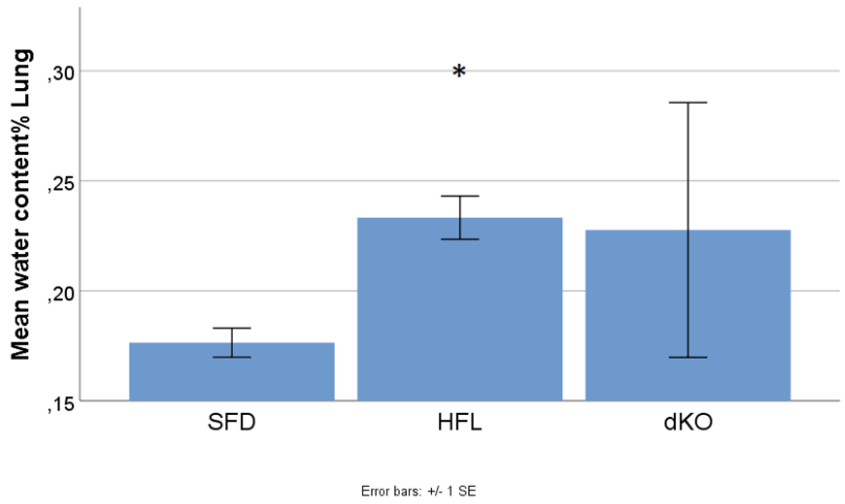

Figure 11. Comparison of mean water content percentage in the lungs between SFD (n=19), HFL (n=16) and dKO (n=3). The asterisk indicates statistical significance. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, dKO – Double knockout group, n – number.*

The mean water content percentage in the kidneys was also significantly greater in the HFL groups in comparison to the SFD groups (Figure 12). HFL M had 3% more water content than SFD M (p = 0.002) and 2.8% more than SFD F (p = 0.005), HFL F had 2.9% more than SFD M (p = 0.005) and 2.7% more than SFD F (p = 0.01).

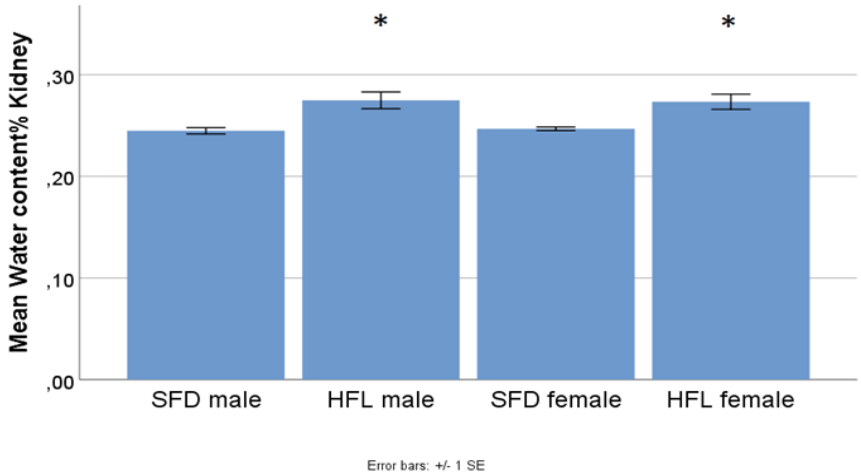

Figure 12. Comparison of mean water content percentage in the kidneys between SFD M (n=10), HFL M (n=8), SFD F (n=9) and HFL F (n=8). The asterisks indicate statistical significance. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F – Female, n – number.*

Body weight

HFL M had a greater body weight mean of 16.1 g than SFD M (p = 3.86 x 10^-10), HFL F had 13.9 g more in comparison to SFD F (p = 2.46 x 10^-8). The males were also significantly heavier than the females (Figure 13).

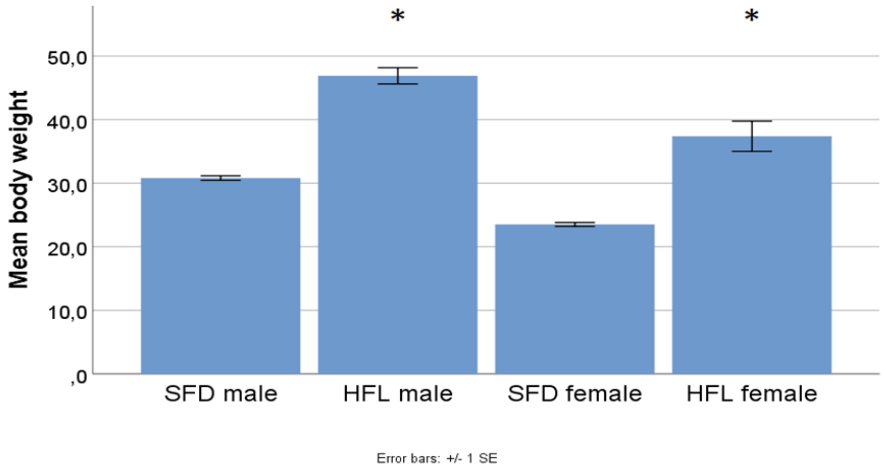

Figure 13. Comparison of body weight in grams between SFD M (n=10), HFL M (n=8), SFD F (n=9) and HFL F (n=8). The asterisks indicate statistical significance. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F – Female, n -number.*

Comparing SFD with obscurin/Obsl1 dKO using ANOVA, there is a significant mean difference of 13.9 g (p = 0.006) even though dKO has a high standard error of 7.41 (Figure 14).

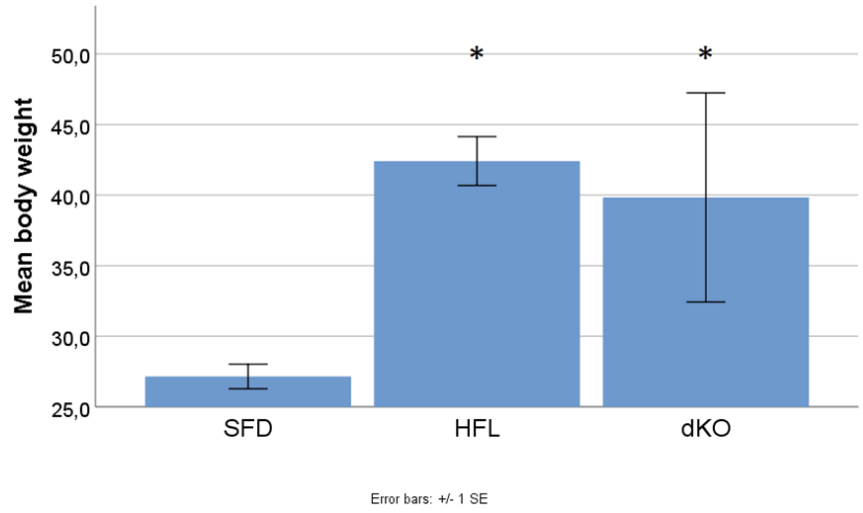

Figure 14. Comparison of body weight in grams between SFD (n=19), HFL (n=16) and dKO (n=3). The asterisks indicate statistical significance. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, dKO – Double knockout group, n -number.*

Tibia lengths

There was no significant difference in tibia lengths between any of the groups (Figure 15 and 16).

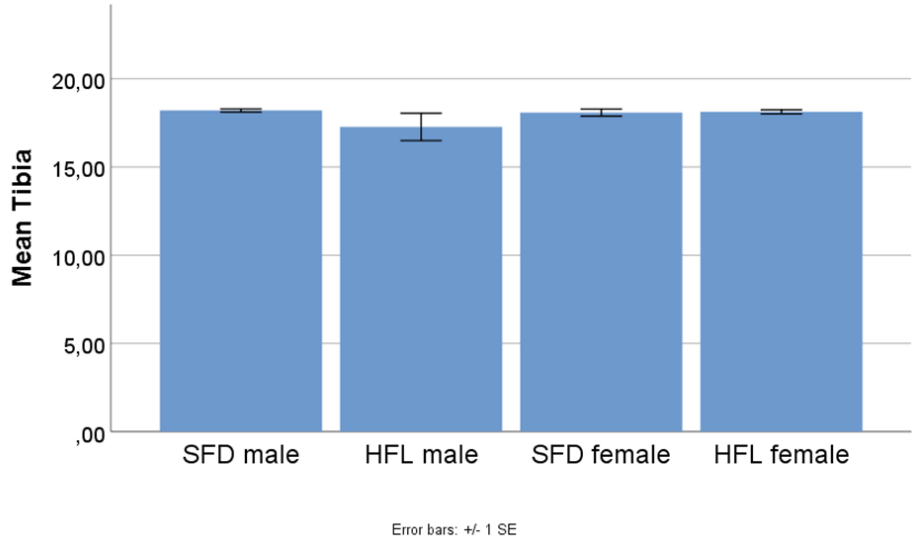

Figure 15. Comparison of tibia length in mm between SFD M (n=10), HFL M (n=8), SFD F (n=9) and HFL F (n=8). No significance is shown. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, M - Male, F – Female, n - number.*

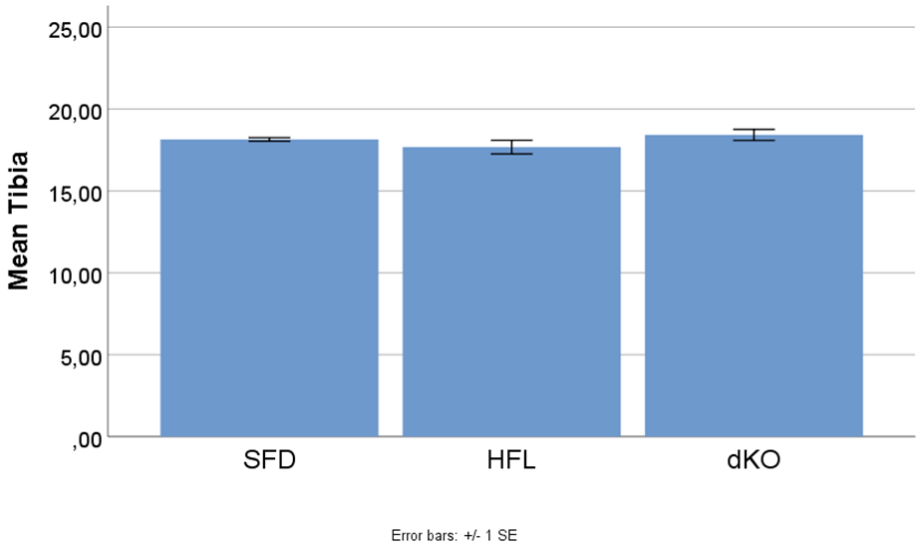

Figure 16. Comparison of tibia length in mm between SFD (n=19), HFL (n=16) and dKO (n=3). No significance is shown. *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, dKO – Double knockout group, n – number.*

Heart failure cells

Table 10 presents the number of HFC found in each group (Figure 17). There is a significant greater amount of HFC in the obscurin/Obsl1 dKO group compared to both HFL ($p = 0.043$) and SFD ($p = 0.045$).

Table 10. The amount of HFC found in 5 different locations of each sample.

Sample ID	Picture 1	Picture 2	Picture 3	Picture 4	Picture 5	Total
SFD1	0	0	0	0	0	0
SFD5	0	0	2	0	0	2
SFD17	0	0	0	0	0	0
SFD18	0	0	1	1	0	2
HFL3	0	0	0	0	0	0
HFL5	0	0	0	0	0	0
HFL11	0	0	1	0	1	2
HFL18	0	0	0	0	1	1
OL2421	3	6	2	1	1	13
OL2471	2	1	1	5	2	11
OL2472	5	11	10	9	12	47

SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, OL – the ID of double knockout mice.

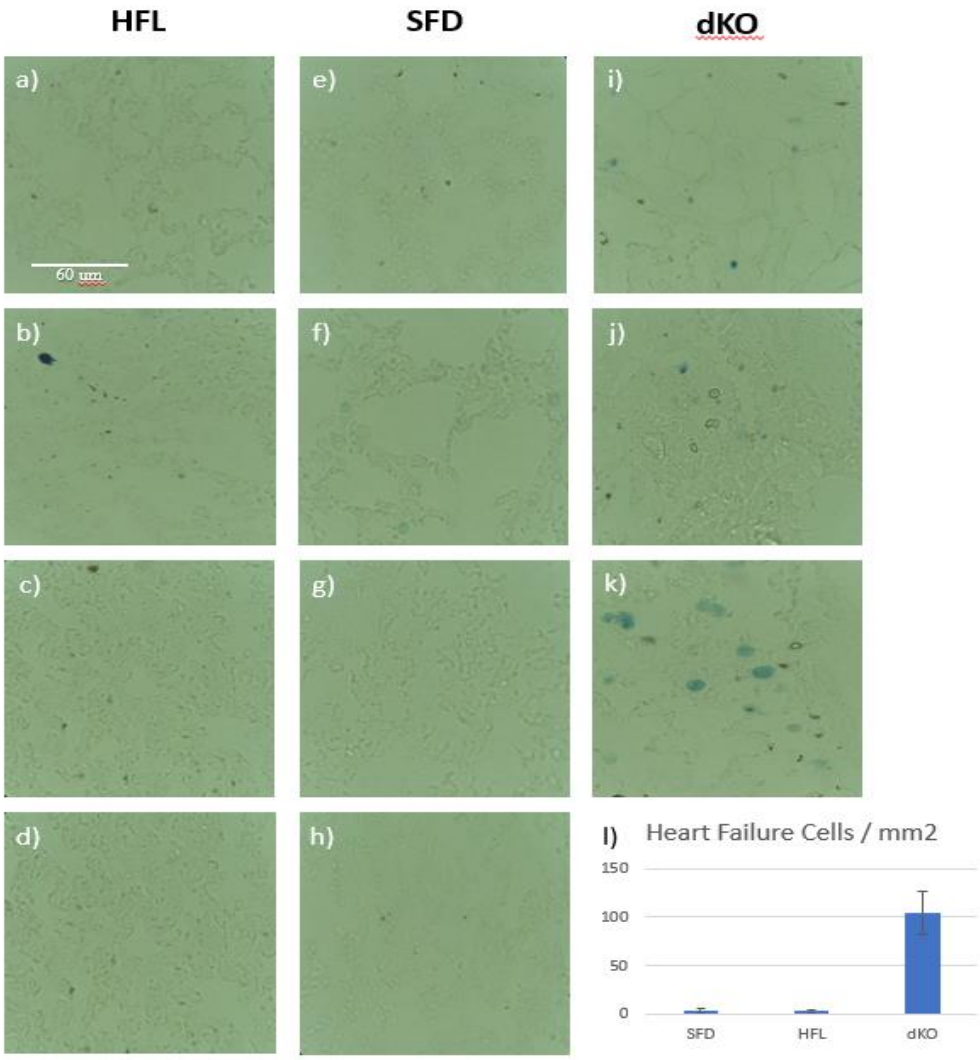

Figure 17. The heart failure cells are the blue cells in the pictures. Picture a) displays a location in HFL 3, b) HFL 5, c) HFL 11, d) HFL 18, e) SFD 1, f) SFD 5, g) SFD 17, h) SFD 18, i) OL 2421, j) OL 2471, k) OL 2472. l) presents the amount of heart failure cells there is per square mm in each group, based on the number of cells in the total area of the pictures. SFD (n=4), HFL (n=4) and dKO (n=3). *SFD - Standard fat diet group, HFL - High fat diet with L-NAME group, OL – the ID of double knockout mice, n- number.*

Discussion

Key findings

In this study, the main purpose was to investigate differences between mice fed with a combination of a high fat diet and L-NAME, a model of HFpEF, and healthy lean mice regarding the expression of cardiac proteins to better understand the factors behind the disease. The results presented showed that there are proteins that differ between the groups, which indicates that changes in certain proteins may be involved in the etiology of HFpEF. It was found that there are significant differences in a total of 56 proteins in the male mice analysis (see Table 7), compared to the 128 proteins in the female mice analysis (see Table 8),

which indicates that more proteins are changed between the female SFD and HFL groups compared to the males. In the combined analysis, most of the significant proteins are only expressed in the female analysis. It is also known that women outnumber men by a 2:1 ratio regarding the disease (5), and that older women with HFpEF experience worse clinical outcomes in comparison to older men (18), but the reason behind the sex difference is unknown. Since the mice were close in age and bred in the same conditions, environment and age are excluded as an explanation to the contributing factors to the difference. There are some epidemiological factors and intrinsic differences between the sexes that are believed to contribute to the number, such as the risk factors being thought to impact women to a greater extent than men in the terms of developing HF (19). Other sex-dependent factors that are thought to be associated with HFpEF are changes in myocyte stiffness, alterations to cardiac calcium handling, the cardiac metabolism and myocardium substrates (ie. free fatty acids, ketone bodies or glucose), low estrogen levels, the renin-angiotensin-aldosterone system, decreasing nitric-oxide during menopause, or progesterone activation of extracellular signal-regulated kinase 2 (18). In addition, reproductive factors, which have shown to play a role in the development of peripartum cardiomyopathy, and breast cancer therapy are thought to be sex-specific risk factors (19). Although there are epidemiological factors and intrinsic differences that are thought to be associated with HFpEF, there are no known explanations to the sex differences, mainly because most of the studies that have investigated treatments in HFpEF patients have not done studies with the aim of researching for differences between the sexes. The proteomic differences between the sexes could indicate that a majority of important heart function genes are only expressed on the Y chromosome, and that no expression is found in females, or that the gene/protein expression is regulated by sex hormones. Sex may also lead to behavioral differences, e.g. males eat more, fight more, get bigger, which could be reflected in differential gene/protein expression. One of the main findings in this study are clear differences in the amount of significantly differentially expressed proteins between sexes, which may serve to help further investigate sex-specific differences. Our study also suggests that sex-specific treatments may be needed when considering therapeutic options. However, further sex-specific investigations are necessary for specific answers for the mechanism of HFpEF and the development of sex-specific HFpEF treatments (5).

Although there are big differences in protein-expression between the male and the female proteome analysis, the mean percentage water content in lungs and kidneys and the amount of HFC in lungs does not differ significantly between the male and female HFpEF mice (see Figure 10 and 12). These findings are not in line with the usual pattern seen in previous studies, with the HFpEF clinical outcomes usually being worse in females than males (18). A possible explanation to this could be that the duration of the disease was not long enough, meaning that if an analysis had been done on the mice after they had been living with the disease for a longer period of time then the results might have been different, and also because the findings of clinical outcomes being worse in females refers to older patients (18). The HFL mice were only 5 months of age when the data was taken, and they had just finished the 16 weeks long L-NAME and high fat diet, meaning that they most likely had not been living with HFpEF for that long. However, confirmation of this theory requires further research with the experimental animals being observed for a longer period of time.

HFpEF proteome analysis

Senataxin (Setx)

Out of the most significant proteins in the combined male and female analysis, only one gene´s expression differs more in the male analysis (FC -8) than in the female analysis (FC -4.64), and that is Setx. It encodes a protein called Senataxin, and it is believed to be a helicase, which unwinds the strands in the DNA or RNA. Senataxin is thought to help with transcription, RNA-molecule processing and repair of damaged DNA. There is only expression of Setx in the SFD group (FC -8), which suggests that a lack of Senataxin could result in HF. However, no cardiac phenotype has been observed in senataxin knockouts. Intriguingly, these mice show aberrant sex chromosome inactivation, suggesting a putative role of the protein in sex-differences observed in HFpEF (20).

S100 Calcium Binding Protein A1 (S100A1)

Some of the differentially expressed proteins in the female analysis already have known functions in cardiomyocytes. S100A1 is only downregulated in the female HFL group (FC -3.84), and previous study has shown that a decreasing S100A1 can result in HF, which correlates with our results. Since the protein is connected to many calcium regulatory proteins such as SERCA2a and ryanodine receptors, it is therefore not surprising that a downregulation would help develop HFpEF considering its importance in regulating the calcium cycling (21).

Matrix metalloproteinase 9 (Mmp9)

Mmp9 is also downregulated in HFL F, and one of its functions is to cleave structural elements of the extra cellular matrix (ECM) such as collagen, which can be found in the ECM in the myocardium. In cardiovascular disease, remodeling of the cardiac muscle tissue is done to preserve the cardiac function with a breakdown of the collagen, a downregulation would therefore mean that this cannot be done optimally (22).

Cat Eye Syndrome Chromosome Region, Candidate 6 (Cecr6/Tmem121B)

Cecr6 (also known as Tmtm121B) is only expressed in the HFL group (FC 8) in both the female and male analysis, suggesting that it could therefore have an importance in the development of HFpEF. Although, there is little to no information about this gene except for that it has been connected to the development of Cat Eye Syndrome, it has been expressed in heart tissue as well (23). Considering that there is only HFL expression of it in both male and female mice, and there is no known function, it could be beneficial to further investigate it.

Lysozyme (Lyz1, Lyz2)

The most significant genes in the combined analysis are the lysozymes Lyz 1 and Lyz 2. Although they are less downregulated in the HFL group in the female analysis (FC -3.84), they are somewhat upregulated in the HFL group in the male analysis (FC 0.88). However, since the change in expression is not much different and the significance is much lower in the male analysis, one could discuss the impact of this change being negligible. They primarily have a bacteriolytic function (24), and no known function involved with HF. However, serum lysozyme has been implicated in impaired glucose metabolism (25), while increased levels of salivatory lysozyme have been associated with hypertension (26). Another possible cardiac-related theory for a deregulation of Lyz1/Lyz2 may stem from the obesity-related increase in systemic tissue inflammation in HFL mice.

Pyruvate dehydrogenase kinase (Pdk4)

Pdk4 is responsible for phosphorylating and inhibiting the enzyme pyruvate dehydrogenase complex (PDC), which plays important function for the cellular metabolism. The PDC stimulates glucose oxidation, meaning that an inhibition of the PDC done by Pdk4 results in a downregulation of glucose oxidation in the cells causing the myocardium to remain heavily dependent on fatty acids as fuel. This is seen in type 2 diabetes mellitus patients due to an increased expression of Pdk4 and it helps with the development of diabetic HF. Cardiac dysfunction can develop due to this change in fuel being associated with accumulation of cardiac myocyte neutral lipid, toxic lipid intermediates and reactive oxygen species (27).

Therefore, since Pdk4 is upregulated in the HFL group in both the males (FC 1.29) and females (FC 1.32), it has most likely had an effect on the development of HFpEF in the mice.

Comparison of proteomes between Obsc/Obsl1 dKO and the HFL model of HFpEF

In both the separate male and female HFL Metascape analysis, cardiac muscle contraction and metabolic processes are the most significantly deregulated biological pathways, and mitochondrial deregulation is also significant, but less (see Figure 5 and 7). While in the Obscurin/Obsl1 dKO analysis, even though deregulation in cardiac muscle contraction and mitochondrial function are seen as well, metabolic processes are far greater significantly changed biological pathways (see Figure 1F). The program Venny is used to see how many of the significantly differentially expressed proteins found in our proteomic study that were also identified in an Obsc/Obsl1 dKO proteomic analysis from a previously done study at the Lange Lab, that has yet to be published. HFL M and dKO have 16 deregulated proteins in common, while HFL F and dKO have 11 proteins in common, and they are all related to metabolic enzymes, mitochondrial enzymes and contractility function. This indicates that these common proteins have an important role in the development of diastolic dysfunction and supports the hypothesis that Obscurin/Obsl1 dKO results in HFpEF.

Analysis of the pre-existing morphometric data

Tibia lengths

There are no significant differences in tibia lengths between the groups (see Figure 15 and 16), meaning that the increased body weight in HFL mice compared to SFD mice was diet-induced and did not affect the growth of the animal. Hence, tibia lengths can be used to normalize other morphometric data, such as body weights.

Pulmonary congestion

Pulmonary edema in HFpEF patients is common due to the fact that the disease gives an elevated cardiac filling pressure which initiates the edema in the lungs (15). This was in line with the results, which showed that there is a 5.68% greater mean water content in the HFL mice compared to the SFD mice ($p = 0.000023$), a result that is comparable to initial reports from this HFpEF animal model (10). There were no significant differences between the sexes regarding pulmonary edema in HFL mice. The dKO mice also had a higher water content percentage, but there was no significance, which is most likely because of the small sample size. There was only data from 3 dKO mice of mixed sex (2 male, one female), which is not

enough data to statistically conclude that a double knockout of obscurin and Obsl1 results in pulmonary edema. To strengthen the hypothesis suggesting that Obscurin/Obsl1 dKO results in HFpEF, a statistical analysis of a larger sample size would be needed.

Renal congestion

The results also showed that there is a significantly higher water content in the kidneys of the HFL mice (Figure 12), which suggests that HFpEF affects the kidneys as well. While chronic kidney disease serves as a risk factor to HFpEF (3), HFpEF could also cause a kidney disease. There is a term called cardio-renal syndrome, meaning that a dysfunction of either the heart or the kidney can induce a dysfunction in the other organ, which falls in line with our results. It is believed that venous congestion is the cause for kidney dysfunction in HF patients (28).

Heart failure cells (HFC)

Surprisingly in this study, HFC were nowhere to be found in the HFL mice while in the dKO mice there were plenty of them even though the sample size was lower compared to the number of HFL and SFD animals studied (Figure 17). Statistical analysis showed significance, because of there being so many cells. An explanation could be that by the time the data of the dKO mice were taken (at about 9 months of age), the mice had been experiencing diastolic dysfunction for at least 3 months, while the HFL mice had their data taken close to the end of their HFpEF-stimulating diet. This suggests that the development of HFC is correlated with the period of time of having the disease, which could further increase the support of the hypothesis that a double knockout of obscurin and obsl1 results in HFpEF. However, since there are no studies on when HFC develop, further research is needed to conclude this statement.

Methodological considerations

One of the strengths of the methods is that parameters for generating and housing the HFL and SFD mice used for the experiment were tightly controlled. They had all been housed in the same environment with always 5 littermate inbred C57/BL6 mice housed together to decrease stress-induced behaviors, fed the same diet during the same period of time and samples were made when the mice were around the same age. This, along with the fact that there are many similar genetic features between humans and mice (17), makes them good test subjects, since factors that could affect the results are excluded.

Another adequate methodological approach is testing out the end-results of the samples in an SDS-PAGE gel prior to them being sent to the Core Facility for mass-spectrometry. By doing so, we can determine if the samples have been made accurately or if they need corrections. It is a waste of both time and money if they are sent to mass spectrometry without having been prepared correctly and therefore display a negative outcome. Although this would have been the adequate approach to our gel-results showing that there was still some myosin left in the MS samples, we concluded that the amount of myosin left was minimal and that it would most likely not affect the results. If a correction were to be done, then it would be an additional centrifugation for the remaining myosin to pellet.

One of the limitations of this study is that SFD animals are also used as a control for Obscurin/Obsl1 dKO mice. While SFD mice are C57/BL6 wildtype mice (Jackson Laboratory, USA) that were on a standard fat diet comparable in caloric content and protein/carbohydrate/fat ratios to the dKO mice, several additional differences need to be taken into account:

1) The dKO strain is in a black-swiss genetic background (29). Although these mice have been derived by crossing inbred C57/BL6 with N:NIH Swiss mice, black Swiss mice are an outbred strain and less susceptible to cardiovascular disease.

2) Owing to the limited number of available dKO mice at the time, these have not been perfectly age- and sex-matched.

3) Control mice should also express the transgenic Nkx2.5-driven Cre recombinase to eliminate any unlikely but potential influence this transgene has on the cardiac and lung phenotype in dKO mice.

Future experiments that utilize age and sex-matched wildtype controls on the same diet will verify if the findings from this pilot study involving differences between HFL and dKO mice hold true.

The small sample size is a limitation, since it decreases the power of a study. Although only 12 samples are used in the proteome analysis, 4 from each group, a statistical significance is

still seen. Meanwhile, when comparing the lung and kidney organ congestion between obscurin/Obsl1 dKO mice and SFD mice, a significant difference is not produced due to the dKO size sample being too small (n=3).

Another limitation is the combined analysis of HFL male with HFL female proteomes. Because the male and female proteome analysis revealed so many differences, it is not suitable to perform analyses that combine results from both sexes due to the variance being too great.

In the SDS-PAGE gel, the bands in the HFL M MS well (Figure 2) were stained stronger than the bands in the other wells even though the protein amounts were normalized and measured (Table 2) prior to the procedure of the SDS-PAGE gel. Possible explanations are inaccurate pipetting and problems with the measurement of proteins using the BioRad Protein Assay kit. However, these limitations did not influence the results from the proteome analysis, as the mass-spectrometry core facility measured/normalized the protein concentrations again.

Pooling is done on the SFD M, SFD F, HFL M and HFL F samples to average out any outliers that may exist within the group. However, a disadvantage is that you cannot look at changes that occur in animals that have mild disease versus animals that have strong disease. Changes in proteins might differ depending on the intensity of the disease, and with pooling, these changes cannot be evaluated.

Conclusions and Implications

The main study set out to identify differences in proteins between healthy lean mice and HFpEF mice to better understand the etiology behind the disease on a proteome level. Many significantly differentiated proteins were identified, mainly in the females. In addition, previous studies have shown a higher prevalence and clinical outcome in female HFpEF patients than in males. Therefore, we suggest that further sex-specific research is needed to explore mechanisms behind the sex differences that could help us understand the pathophysiology and thereby identify treatments for this condition, and the differences

between the sexes´ proteomes might serve as help in the development of these sex-specific treatments.

When comparing the proteomes and the pathway analysis between the Obscurin/Obsl1 dKO and the HFL mice we find that most proteins that are found in both models are involved in the cardiac metabolism, mitochondrial structure/function or in cardiac contractility. This supports the hypothesis that the significant deregulation of metabolic enzymes, mitochondrial proteins and proteins involved in cardiac contractility observed in the Obscurin/Obsl1 dKO model may play a role in the development of HFpEF. Further investigation that continues to delineate similarities and differences between both HFpEF models is encouraged.

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Populärvetenskaplig sammanfattning

En analys kring skillnader i diverse fynd och proteiner i hjärtan hos möss med hjärtsvikt med bevarad systolisk funktion.

Hjärtsvikt är ett tillstånd där hjärtat inte kan pumpa ut tillräckligt med blod för att kroppens organ skall försörjas med rätt mängd syre och näring, vilket kan ge symptom som andfåddhet, trötthet och bensvullnad. Hjärtsvikt kan delas in i olika grupper beroende på vad som orsakar det. I detta arbete fokuserade studien på hjärtsvikt med bevarad systolisk funktion, förkortad HFpEF. Vid HFpEF är felet att fyllnaden av blod in i hjärtats kamrar är otillräcklig p.g.a. stelhet i hjärtmuskelväggen, vilket bidrar till att hjärtsvikt uppstår trots en normal pumpförmåga. Det finns ingen optimal behandling till denna typ, då dess bakomliggande orsaker är än idag okända.

I det här arbetet fokuserade studien på att analysera och jämföra diverse kliniska fynd samt proteiner som finns i hjärtan hos möss med HFpEF, friska möss och möss som tidigare fått generna Obscurin och Obscurin-like 1 avstängda via genetisk modifiering vilket vi kallar för en ”double knockout” (dKO), då dessa tidigare visat sig ge liknande tecken som setts hos HFpEF patienter. Detta görs för att man skall komma ett steg närmare till att förstå sig på bakomliggande mekanismer och orsaker till sjukdomen.

Vi såg att HFpEF mössen hade större påverkan på lungor och njurar med en markant ökat mängd vätska i dessa organ jämfört med de friska mössen. dKO mössen hade också en ökad mängd vätska, men eftersom vi endast hade tillgänglig data från ett fåtal dKO möss till skillnad från HFpEF och friska möss, så kunde denna resultat inte uteslutas från att vara en slump. Vi fann väldigt få så kallade ”heart failure cells” (d.v.s. celler som hittas i lungor hos de med hjärtsvikt) hos friska och HFpEF möss, men däremot väldigt många hos dKO mössen trots att antalet på dessa var en färre än de förstnämnda, vilket tros kunna bero på hur länge mössen var sjuka innan datan togs. dKO mössen hade levt längre med sjukdomen än HFpEF mössen. Vi såg även att HFpEF honorna hade många fler förändrade proteiner än HFpEF hanarna, vilket stödjer påståendet om att kvinnor drabbas hårdare av sjukdomen. Jag skriver även om några av dessa proteiner och deras potentiella betydelse i utvecklingen av sjukdomen. De nedsatta proteinerna hos HFpEF mössen gav även nedsättning i samma typ av funktion som de nedsatta proteinerna man funnit hos dKO möss i en tidigare opublicerad

studie, vilket stödjer idén om att en double knockout av generna Obscurin och Obscurin-like 1 faktiskt ger HFpEF. Dessa påståenden kräver dock ytterligare utredning för att kunna säkerställas.

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