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Da Young Lee April 19, 2020

Growth Differentiation Factor 15 (GDF15) Causes Cardiac Cachexia in Heart Failure

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Background & Aims

Cachexia is the wasting of normal body tissues and occurs in chronic medical diseases. It is a common complication of heart failure (HF) that is associated with high mortality. Growth differentiation factor 15 (GDF15) regulates food intake and can cause cancer cachexia. GDF15 is a sensitive biomarker in human HF, though its biologic function in HF is unknown. This study investigates the role of GDF15 in HF using a genetic mouse model of dilated cardiomyopathy (DCM).

Methods

Q-PCR and ELISA were performed to assess the expression, tissue distribution and circulating levels of GDF15 in DCM and age-matched wild type (WT) mice. A double transgenic mouse was created by crossing our DCM model with a constitutive Gdf15 knock-out (KO). Using this novel model, we quantified food intake and assessed fat and lean tissue mass by direct tissue weights at necropsy and by dual-energy X-ray absorptiometry (DXA). Cardiac function was assessed using echocardiography, and histochemistry performed to quantify cardiac fibrosis. Survival was assessed by Kaplan-Meier.

Results

GDF15 mRNA (43-fold; $p<0.01$) and protein (54-fold; $p<0.01$) were increased in LV tissue, and circulating GDF15 was elevated (8.3-fold; $p=0.03$) with advanced DCM. *Gdf15* mRNA was not increased in any other organs. DCM mice developed cachexia as assessed by reduced fat and lean mass by tissue weight and reduced fat mass by DXA in association with reduced food intake (vs. WT; $p<0.01$ for all). DCM mice with *Gdf15* KO had preserved fat and lean tissue mass and consumed more food ($p\leq0.01$ for all) than regular DCM mice. *Gdf15* KO had no effect on cardiac structure or function by echocardiography and KO mice displayed only a small reduction in cardiac fibrosis relative to regular DCM mice (3%; $p<0.01$). Despite this, *Gdf15* KO prolonged survival in DCM mice (29±3 vs. 25±3 weeks; $p<0.01$).

Conclusions

GDF15 is a novel cardiac hormone produced in HF that triggers anorexia and cachexia in HF by an extra-cardiac mechanism.

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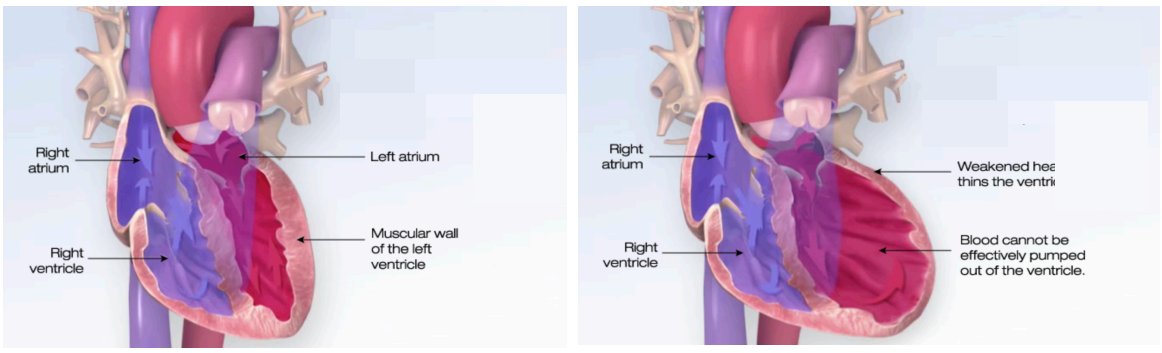
Table of Contents

INTRODUCTION

The heart is a highly specialized muscle that begins beating at 6-weeks' gestation and does not stop until the time of death. This remarkable feat of evolution allowed for the development of larger and larger organisms as the heart circulates blood and nutrients continuously throughout the body. The human heart is composed of 4 chambers; the top 2 chambers (called atria) receive blood from different regions of the body and pump this blood into the 2 bottom chambers. These bottom chambers (called ventricles) pump blood around the body. The left ventricle (LV) is the main pumping chamber of the heart and circulates blood throughout the entire body, while the right ventricle (RV) pumps blood to the lungs where it picks up oxygen (Fig.1).[1] Normal heart function is critical to life; therefore, it is not surprising that any conditions that damage the heart are potentially life threatening. These include acute processes such as myocardial infarction (MI) or cardiac infections, as well as chronic diseases such as ischemic heart disease and dilated cardiomyopathy (DCM).[2,3] These highly disparate conditions damage the heart function, leading to a common syndrome called heart failure (HF).



(https://heart.org/en/health-topics/heart-failure/)

Figure 1. Heart failure is a complex, chronic condition. Compared to **(A)** the normal heart, **(B)** HF patients have dilated ventricles with thinned and weakened muscular walls. (American Heart Association, 2021).

HF is a complex syndrome that can result from any cardiac disorder that impairs the ability of the ventricles to fill or eject blood.[3,4] HF patients commonly experience shortness of breath and fatigue, both of which reduce quality of life substantially. This includes difficulty with simple daily activities such as walking and climbing stairs. HF is one of the leading cause of morbidity and mortality in the United States and the world. Approximately 6.2 million adults in the U.S. have HF.[5] Although many pharmacological and non-pharmacological treatments have proven effective for the treatment of HF, mortality remains very high. After a new diagnosis of HF, mortality is 10% at 30 days, 22% at 1 year, and 52% at 5 years.[5,6] Notably, HF has a mortality rate worse than nearly all common forms of cancer (Table 1),[5] and a majority patients experience some degree of chronic functional limitation.

Disease	Prevalence*	5-year Mortality
Heart failure	**6.2 million**	**52.6%**
Cancer (all types)	**15.8 million**	**32.6%**
Breast cancer	3.58 million	10.0%
Prostate cancer	3.17 million	2.2%
Colorectal cancer	1.35 million	35.4%
Melanoma (skin)	1.25 million	7.3%
Thyroid cancer	859,838	1.7%
Endometrial/uterine cancer	793,846	18.8%
Bladder/urothelial cancer	712,614	23.1%
Non-Hodgkins lymphoma	719,831	27.3%
Lung cancer	*558,250*	*79.5%*
Renal cell cancer	558,023	24.8%

(http://seer.cancer.gov/statfacts/)

Table 1. Disease prevalence in the United States.

As LV dysfunction develops, many compensatory mechanisms are activated including the renin-angiotensin-aldosterone and sympathetic nervous systems.[7,8] These compensatory mechanisms initially serve to stabilize and maintain normal cardiac output. However, as HF progresses, these systems become detrimental and are a key driver of HF progression. Abnormal

LV function also leads to systemic complications such as fluid retention (e.g., pulmonary edema, ascites, leg swelling), kidney and liver dysfunction, and altered systemic metabolism.[9] Late stage disease is marked by cardiac output that is insufficient to meet even basal metabolic demands (called cardiogenic shock). This is associated with severe functional limitations, anorexia, and fatigue, with some individuals developing total body wasting, termed cardiac cachexia.[3-4,7-8]

Cardiac cachexia has been recognized as a component of HF for centuries, with the earliest characterization ascribed to Hippocrates who wrote "...the shoulders, clavicles, chest, and thighs melt away…" when describing individuals with dropsy, an ancient term for HF.[10] Cachexia is defined as unintentional edema-free weight loss of >7.5% of body weight over a 6-12 month period, or a body mass index (BMI) <20kg/m^2 with physical and biochemical evidence of wasting.[11] Cachexia is present in up to 40% of patients with HF[12] and is associated with high mortality of 50% at 18-months.[13] Cachexia becomes more prevalent as HF progresses, being found in up to 60% of those awaiting heart transplant.[14] Cachexia is also an independent predictor of death,[15] and patients with more severe cachexia fare proportionally worse.[15] Finally, pre-operative cachexia is a risk factor for death after heart transplant.[16,17]

Despite these morbid data, little is known of the molecular mechanisms that drive the transition to a cachectic state in some HF patients. Cardiac cachexia has been attributed to alterations in a multitude of seemingly disparate systemic processes that are common in HF including neurohumoral imbalance, changes in appetite, metabolic dysregulation, and sarcopenia (Fig. 2).[18] These changes contribute to a catabolic-anabolic imbalance resulting in progressive protein and fat degradation, resulting in cachexia. Some HF patients also develop anorexia and malnutrition that may also contribute. No intrinsic cardiac mechanisms are known to cause cachexia in HF.

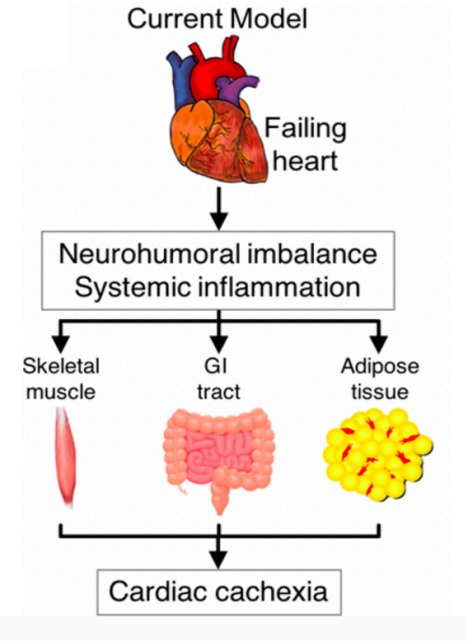


Figure 2. Postulated mechanisms of cardiac cachexia in HF. Cachexia is believed to be a consequence of many disparate pathophysiologic processes common in HF that affect multiple tissues, resulting in a net catabolic state that ultimately results in cachexia. However, the molecular mechanisms directly relating to cachexia are not known.

Like HF, cancer is a chronic disease that frequently leads to cachexia. In a mouse model of prostate cancer, a tumor-produced cytokine called macrophage inhibitory cytokine-1 (since renamed growth differentiation factor 15; GDF15) was found to trigger cachexia.[19] Mice with a tumor overexpressing GDF15 developed anorexia that eventually led to cachexia as evidenced by reduced fat and lean body mass that was reversed by treatment with a GDF15 neutralizing antibody. In humans with prostate cancer the levels of circulating GDF15 correlate with cancer-associated weight loss and were found to be an independent predictor of cachexia.[19] These data identified GDF15 as a putative disease-specific regulator of cachexia.

GDF15 is a distant member of TGFβ growth factor superfamily. It is a stress-induced cytokine predominantly expressed in the liver, but it can also be produced by other organs in response to variety of stressors. Previous studies have shown that GDF15 is released in response to tissue injury. *Gdf15* mRNA expression in the liver increased dramatically and rapidly following various chemical and surgical treatments that caused acute liver.[20] In murine models of both kidney and lung injury, markedly upregulated GDF15 was also identified.[21] Increased GDF15 serum

levels have a strong association with other diseases including cancer, HF, inflammatory syndromes (e.g, arthritis, rheumatologic diseases, etc...), and obesity.

Animal models have revealed that GDF15 regulates appetite. Transgenic mice overexpressing *Gdf15* have a persistently lean phenotype, while *Gdf15* KO mice have higher weight and increased adiposity compared to wild type (WT) mice due to a change in food intake.[19, 22-24] Unlike other hypothalamic hormones or leptin, GDF15 levels are not responsive to short term changes in caloric intake in mice or humans.[24] However, it has been found that GDF15 levels increase with chronic nutritional stress and after ingestion of a toxin such as chemotherapy.[25]

GDF15 regulates food intake by activating hypothalamic neurons via GDNF-family receptor-like-α (GFRAL), which is a cell-surface receptor that is expressed exclusively in a discrete region of the hindbrain called the area postrema.[26,27-29] The interaction between GDF15 and GFRAL is highly specific, with no other receptors found to have the capability of binding to GDF15 (in >4000 screened).[26,27-29] Upon binding GFRAL, GDF15 suppresses food intake and regulates body weight by inducing subsequent activation and phosphorylation of its co-receptor Ret and phosphorylation of signaling molecules including Erk1/2, Akt, and PLCγ.[30]

GDF15 is an exquisitely sensitive biomarker. Elevated levels predict incident cardiovascular events including MI, new onset HF, and stroke in healthy individuals.[31-38] It is similarly predictive in those with stable coronary artery disease,[39-43] acute coronary syndromes,[44-49] congenital heart disease,[50] atrial fibrillation,[51,52] and HF (Fig. 3),[53-58] as well as many other chronic non-cardiac diseases. In patients with HF, higher GDF15 levels correlate with a greater burden of symptoms[55,56] and with both lower ejection fraction (EF)[55] and lower cardiac output.[57] Interestingly, GDF15 is inversely correlated with BMI in HF patients,[55-57] suggesting a link between GDF15 and body weight in HF.[55-57]

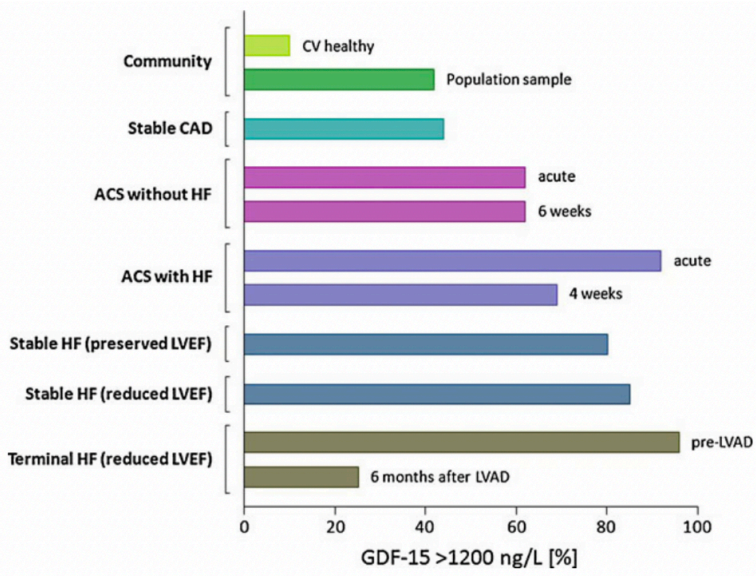


Figure 3. GDF15 as a prognostic biomarker in numerous diseases. Data from numerous studies of serum GDF15 levels in distinct populations of individuals ranging from healthy to those with end-stage HF were collated to assess for the percent of study individuals with GDF15 levels that were above 1,200 ng/L, which is the upper limit of the reference interval. Circulating GDF15 levels increase with disease severity and predict poor outcomes.
CV= cardiovascular; CAD= coronary artery disease; ACS= acute coronary syndrome; LVEF= left ventricular ejection fraction; LVAD= left ventricular assist device. (from: Wollert et al., 2012).[58]

Despite this, the biological role of GDF15 in HF remains unclear. In a knock-out (KO) mouse model subjected to MI, GDF15 was shown to be protective by limiting infarct size.[59] GDF15 KO mice suffered larger infarcts leading to cardiac rupture and significantly higher mortality. Interestingly, the mechanism of benefit was extra-cardiac: GDF15 produced by the heart limited excessive chemokine-activated leukocyte arrest on the endothelium by counteracting chemokine-triggered conformational activation and clustering of β2 integrins on leukocytes, thereby reducing excessive inflammation and cardiac rupture.[60] Additionally, GDF15 was shown to be cardio-protective in pressure overload cardiomyopathy induced by transverse aortic constriction (TAC).[61] Overexpression of GDF15 attenuated cardiomyocyte hypertrophy after TAC, whereas GDF15 KO led to enhanced cardiac hypertrophic growth. Hence, in the setting of an *acute* cardiac insult, GDF15 may be cardio-protective by an as-yet-defined mechanism.

Given the unknown effects of chronic GDF15 expression in HF, I chose to use a genetic mouse model that develops slowly progressive DCM over 5-6 months, thus more closely

mimicking human HF. Our lab uses a transgenic model that expresses a missense mutation (Arg → Cys) at residue 9 (R9C) in the phospholamban (*Pln*) gene.[62] PLN is a transmembrane phosphoprotein that disrupts the cardiomyocyte sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase (SERCA2a) pump. This results in abnormal cardiomyocyte calcium cycling, which is a common feature of nearly all forms of HF, making this model widely applicable.

The PLN$^{R9C/+}$ mutation consistently results in progressive DCM, HF and premature death or heart transplantation in humans before the age of 40.[62-64] The transgenic PLN$^{R9C/+}$ mouse model recapitulates all of these hallmark features of HF: cardiac dilation, reduced EF, fibrosis, inflammatory network activation, and myocardial metabolic derangement (Fig. 4).[62,65] PLN$^{R9C/+}$ mice display normal cardiac structure and function until 10-weeks of age (preDCM phase), develop severe DCM by 18-weeks, and overt HF by 25-weeks of age.

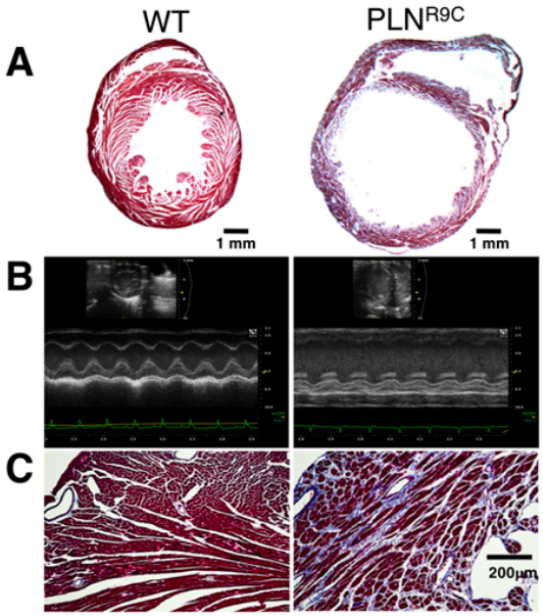


Figure 4. PLN$^{R9C/+}$ mice develop cardinal features of HF. (A) Whole mount LV sections show marked biventricular dilation in PLN^{R9C} mice. **(B)** Representative echocardiographic images show ventricular dilation and reduced cardiac function. **(C)** LV tissue sections stained with Masson trichrome demonstrates severe fibrosis (blue) in PLN$^{R9C/+}$ mice. (modified from: Burke et al., 2016).[65]

PLN$^{R9C/+}$ mice also develop substantial cachexia at the end of life (Fig. 5).[11,26,58]

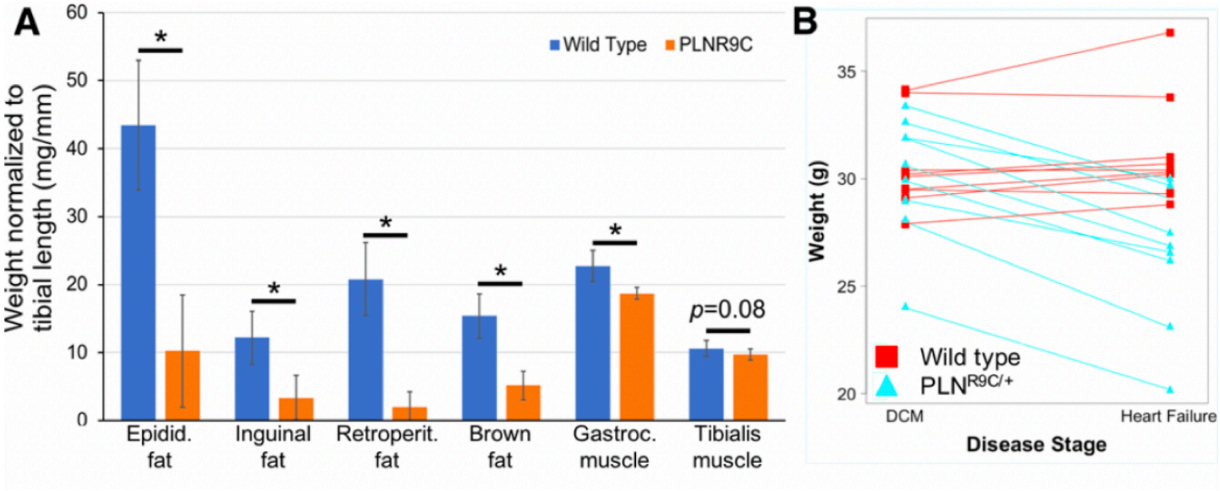


Figure 5. PLN$^{R9C/+}$ mice develop cardiac cachexia. (A) Selected tissues were dissected from PLN^{R9C} mice with HF or age-matched WT mice. Tissue was weighed and normalized to tibial length (*p<0.001, n=10). **(B)** Cardiac cachexia was also reflected by weight loss in PLN^{R9C} mice with overt HF (p=0.003, n=10). (Burke et al., 2016).[65]

Using high-throughput next-generation RNA-sequencing (RNA-seq), our lab incidentally identified a marked ~40-fold upregulation of *Gdf15* mRNA in PLN$^{R9C/+}$ hearts with established, severe DCM.[65] To assess whether *Gdf15* upregulation was common to other mouse HF models, we reviewed data in the NCBI's gene expression omnibus (GEO) database, a repository of high-throughput gene expression and other functional genomics data. This confirmed that GDF15 is substantially upregulated in the heart in a wide array of mouse models of HF (Table 2).[10,59,65-71] However, we note that none of these studies examined the biologic effect of GDF15 in the heart. In this study, my aim was to explore a paradigm shifting idea: GDF15 is a cardiac-derived hormone that causes cardiac cachexia. **Our overarching hypothesis is that GDF15 is a hormone secreted by the failing heart that causes anorexia and cardiac cachexia.**

Model	Fold Change	Method
Genetic DCM	N. R.	Microarray
MI & I/R	10-20	Microarray
Genetic HCM	2.7	RNA-seq
Genetic DCM	26	RNA-seq
Genetic DCM	30-40	RNA-seq
TAC	15	RNA-seq
MI	13	RNA-seq
MI	9.2	RNA-seq
Genetic DCM	35	RNA-seq

Table 2. GDF15 is upregulated in cardiomyopathy of varying etiologies. Data was extracted from manuscripts or the Gene Expression Omnibus. DCM=dilated cardiomyopathy; MI=myocardial infarction; I/R= ischemia/reperfusion; HCM= hypertrophic cardiomyopathy; TAC= thoracic aortic constriction; N.R.= fold change not reported, but significantly elevated

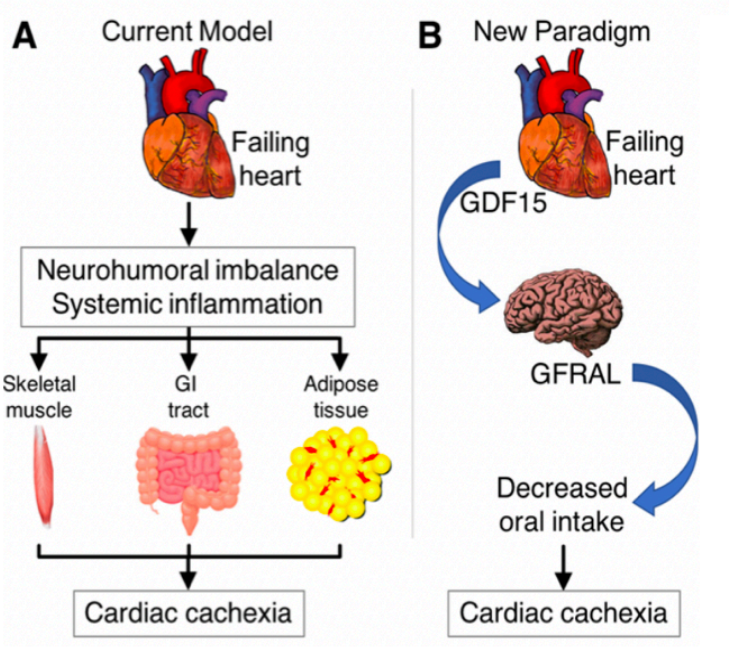


Figure 6. Postulated mechanisms of cardiac cachexia in HF. (A) Cachexia is believed to be a sequelae of chronic neurohumoral and inflammatory dysregulation in HF. **(B)** We propose a novel paradigm: GDF15 is a myocardial-derived hormone that acts centrally via GFRAL in the hindbrain to decrease oral intake and cause cachexia.

METHODS

Mouse Models

The transgenic $PLN^{R9C/+}$ mouse model is established in the FVB genetic background, and has been previously described.[64] To assess the role of GDF15 in HF, we obtained a whole-body, constitutive *Gdf15* KO (KOMP allele $Gdf15^{tm1a(KOMP)Wtsi}$) mouse model that eliminates GDF15 expression from the time of early embryogenesis in all tissues. *Gdf15* KO mice were obtained in the black-6 genetic background and were crossed for 10 generations to FVB mice thereby generating incipient congenic *Gdf15* KO mice in the FVB genetic background. *Gdf15* KO mice were crossed with $PLN^{R9C/+}$ and 4 cohorts of mice were assessed: (1) $Pln^{+/+}$-$Gdf15^{+/+}$ - WT mice; (2) $Pln^{+/+}$-$Gdf15^{-/-}$ - otherwise normal mice lacking *Gdf15*; (3) $Pln^{R9C/+}$-$Gdf15^{+/+}$ - DCM mice that express *Gdf15*; (4) $Pln^{R9C/+}$-$Gdf15^{-/-}$ - DCM mice lacking *Gdf15*. Studies were approved by the Emory University Institutional Animal Care and Use Committee.

Quantitative-PCR: mRNA was extracted from heart, lung, spleen, brain, kidney, liver, muscle, fat, small intestine, and colon tissue using TRIzol, and then converted to cDNA (SuperScript III First-strand Synthesis System, Invitrogen). Q-PCR was performed using VIC- or FAM-labeled TaqMan probes on a BioRad QX200 digital droplet PCR platform. 25µl of the reaction mixture containing 1µl of cDNA solution, 12.5 µl of digital PCR™ Supermix (Bio-Rad), 1.25 µl of GDF15 probe, and 10.25 µl of DEPC H_2O was loaded into a 96-well PCR plate with 70 µl of QX200 Droplet Generation oil (Bio-Rad) and then placed into QX200 Droplet Generator (Bio-Rad). The generated droplets from each sample were transferred to a new 96-well PCR plate, and PCR amplification was performed on a thermal cycler at 95℃ for 10 min, followed by 40 cycles of 95℃ for 30 seconds and 60℃ for 1 min, 1 cycle of 98℃ for 10 min, and finally ending at 4℃.

The plate was then loaded into the QX200 Droplet Reader (Bio-Rad) followed by data analysis to define gene expression. In parallel, Q-PCR was performed using 18sRNA probe for all cDNA tissue samples. All tests were performed in duplicate (technical replicates). *Gdf15* levels were normalized to 18sRNA.

ELISA: Mice were heavily anesthetized with isoflurane vaporizer (VetEquip). Under continuous anesthesia, hearts were exposed by midline thoracotomy and whole hearts extracted. Hearts were then washed in PBS to remove blood and the atria, great vessels and RV were resected. Protein was isolated from LV tissue using RIPA buffer. Serum samples were extracted under heavy anesthesia via terminal exsanguination using a subxiphoid, direct cardiac puncture approach. Quantikine ELISA Kit (Mouse/Rat GDF-15 immunoassay) was used following the manufacturer's directions on extracted protein and serum samples. All samples (50µl) were incubated in GDF15 antibody-coated microplates for 2 h, then washed and incubated with GDF15 antibody conjugate for 2 h. After washing, color reagent was added and the plate was incubated for 30 min. The optical density of each well was determined using a microplate reader set to 450 nm and 570nm. The subtract readings at 570 nm from the readings at 450 nm corrected for optical imperfections in the plate. The standard curve was generated with Calibrator Diluent RD5P diluted at 1:5 (500 pg/mL standard serving as the high standard), and GDF15 pg/mg protein and GDF15 pg/ml were respectively found for LV tissue and serum. All tests were performed in triplicate (technical replicates).

Tissue Weight: Mice were anesthetized with isoflurane upon developing visible symptoms of overt HF (visible panting; reduced activity; reduced/absent recoil from human touch). Fat pads

(inguinal, epididymal, retroperitoneal, interscapular) and muscle tissue (gastrocnemius, tibialis) were extracted at necropsy and immediately weighed for all 4 cohorts of *Pln-Gdf15* mice. Tissue weight was normalized to tibial length, which is standard in HF studies as mice develop varying amounts of edema, making normalization to body weight unreliable.

Dual energy X-ray absorptiometry (DXA): At 24-weeks of age, mice were anesthetized with isoflurane and DXA scanning was performed using the Kubtec Scientific Parameter Cabinet X-ray System. Soft tissue body composition (fat and lean/muscle mass) was assessed. Fat and lean mass were normalized to tibial length.

Food Intake: All 4 cohorts of *Pln-Gdf15* mice were individually housed from age 18-weeks and total food intake was quantified by weight weekly. Once overt HF was identified, mice were sacrificed; WT mice were sacrificed in matched fashion to *Gdf15* KO status. Data were normalized to tibial length.

Survival: A cohort of $Pln^{R9C/+}$-$Gdf15^{+/+}$ and $Pln^{R9C/+}$-$Gdf15^{-/-}$ were followed to assess survival. Survival was defined as survival free of overt HF or death, with overt HF as defined above.

Echocardiography: Mice were anesthetized with isoflurane and attached to ECG leads on a Vevo Mouse Handling Table (VisualSonics InC.). Chest hair was removed with depilatory cream. Transthoracic echocardiography was performed with heart rate approximately 500-550 bpm using

a Vevo 770 High-Resolution In Vivo Micro-Imaging System and RMV 707B scan-head (VisualSonics Inc.). Images were obtained by a single, experienced echocardiographer blinded to genotype. Parasternal 2D and M-mode images were acquired to assess LV wall thickness (LVWT), LV end diastolic diameter (LVEDD), LV end systolic diameter (LVESD), and fractional shortening (FS) (calculated as FS = [LVEDD-LVESD]/LVEDD).

Histochemistry: Hearts were extracted as described above under heavy anesthesia. Hearts were washed in PBS, fixed in 4% paraformaldehyde overnight at 4°C, and then washed 2 times in 1X PBS for 10min. Fixed hearts were then embedded in paraffin and sectioned at 5μm. Slides at approximately 100μm increments from base to apex were stained with picrosirius red to identify LV fibrosis. Slides were imaged using a Hamamatsu NanoZoomer SQ, analyzed by Image J (NIH) and data expressed as fibrosis percent of total LV area.

Statistics: Data are presented as mean ± SD for normally distributed data. For QPCR, ELISA, tissue weight, body composition by DXA scan, and food intake data, between-groups differences were calculated using a 2-tailed Student's *t* test; *P* values less than 0.05 were considered significant. For picrosirius red-stained fibrosis data, between-groups differences were calculated using a 2-tailed Student's *t* test for single paired comparisons; *P* values less than 0.05 were considered significant.

Survival data were analyzed using the log-rank test. A *p*-value less than 0.05 was considered significant. To estimate sample size for survival data, a power calculation was performed using the "pwr" package in R ("pwr.chisq.test") on our prior survival data in PLN^{R9C} mice. To have 90% power to detect a moderately large effect size (i.e., improved survival with

Cohen's w statistic w=0.4), the estimated sample size was at least n=33/genotype. For echocardiography, time-dependent between-groups variance was calculated using ANOVA with a model-based fixed-effects standard error method. To estimate sample size, a power calculation was performed based on our prior echo data in $PLN^{R9C/+}$ mice using the "pwr" package in R. To have 90% power to detect a 50% improvement in FS and LVEDD (a moderate effect size with Cohen's f statistic f=0.5) across 4 groups of mice at α=0.05, we needed n=16 mice/group. Since $PLN^{R9C/+}$ mice can die prematurely at any point, we used 17-18 $PLN^{R9C/+}$ in each arm. Because WT mice are known to display little variance in cardiac structure and function in this age range, sample size was reduced to n=10 in these cohorts to limit unnecessary mouse usage. Tests of the echocardiographic hypotheses were conducted using Bonferroni's corrected *P* values for significance (i.e., FDR<0.0125).

RESULTS

GDF15 is upregulated in PLN$^{R9C/+}$ hearts.

My first aim was to determine the expression patterns of *Gdf15* in the PLN$^{R9C/+}$ model. Cardiac *Gdf15* increases as DCM progresses in PLN$^{R9C/+}$ mice. In the preDCM phase, cardiac *Gdf15* mRNA levels were ~6-fold upregulated (p-value); with progression to advanced DCM, cardiac *Gdf15* mRNA levels were ~40-fold upregulated (p-value).[65] Interestingly, when heart tissue was separated into cardiomyocyte and cardiac non-myocyte cell fractions, *Gdf15* was specifically induced in PLN$^{R9C/+}$ cardiomyocytes (21-fold, $p<0.01$), with no significant increase found in non-myocytes.[65]

To determine the expression and tissue distribution of *Gd15* in PLN$^{R9C/+}$ mice, we performed QPCR in different organ tissues from preDCM PLN$^{R9C/+}$ mice, mice with advanced DCM, and age/sex-matched WT. PreDCM PLN$^{R9C/+}$ mice displayed 10-fold higher *Gdf15* levels in the heart (n=4/genotype, p=0.17; Fig. 7A), while mice with advanced DCM displayed 43-fold higher *Gdf15* levels compared to matched WT, confirming previously published results (n=4/genotype, $p<0.01$; Fig. 7B). *Gdf15* levels were low and did not increase significantly in other organs in PLN$^{R9C/+}$ mice compared to WT at either time point (Fig. 7). This shows that *Gdf15* is specifically and exclusively upregulated by the heart in PLN$^{R9C/+}$ mice as DCM progresses.

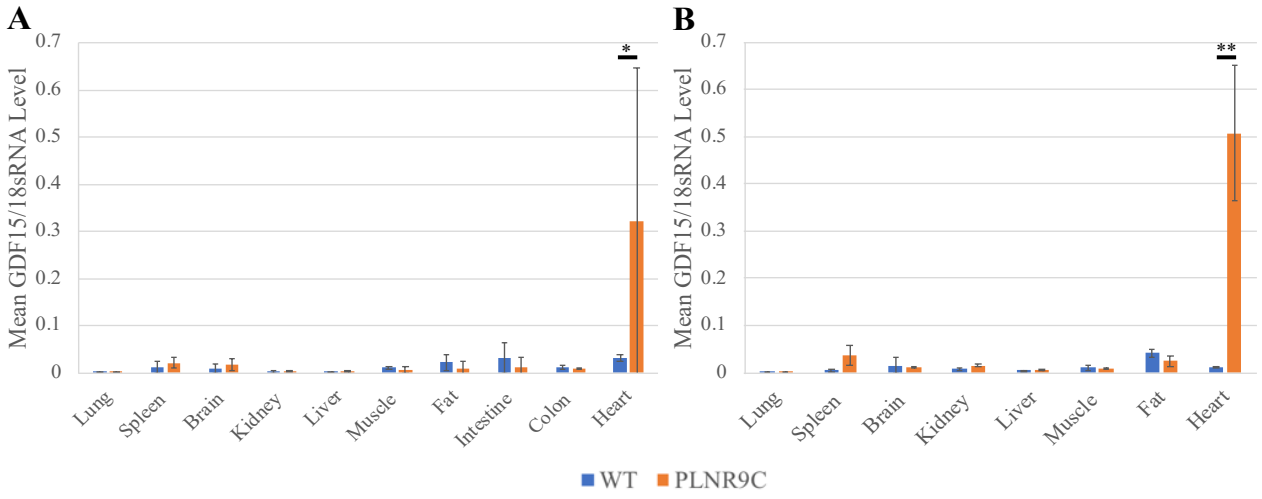


Figure 7. Mean *Gdf15* normalized level is upregulated in PLN$^{R9C/+}$ hearts. (A) Nonsignificant increase in *Gdf15* mRNA levels was observed at 8 weeks of age (preDCM) (*p=NS). **(B)** Significant increase in *Gdf15* mRNA levels at was observed at 22 weeks of age (DCM) (**p<0.01). N=4 mice/genotype; *t* test.

To confirm that this increased mRNA expression correlated with increased GDF15 protein in the heart and blood stream, I performed ELISA on LV tissue and serum from preDCM PLN$^{R9C/+}$ mice, those with advanced DCM, and age/sex-matched WT. In preDCM LV tissue, GDF15 was significantly higher than in WT hearts, where GDF15 was barely detectable (28-fold, n=4/genotype; p=0.01). Similarly, GDF15 was significantly higher in LV tissue from mice with advanced DCM (54-fold, n=4/genotype, p<0.01 Fig. 8A). Myocardial GDF15 levels increased 11-fold with progression from preDCM to advanced DCM (p<0.01). Circulating GDF15 was not detected in 8-week old mice. With progression to advanced DCM, circulating GDF15 increased substantially compared to preDCM mice (298 pg/ml in DCM PLN$^{R9C/+}$ mice, n=4/genotype, p<0.01; Fig. 8B). These data confirm that myocardial GDF15 levels increase with DCM progression and demonstrate that GDF15, which is upregulated by the failing heart, is secreted to high levels in the blood stream.

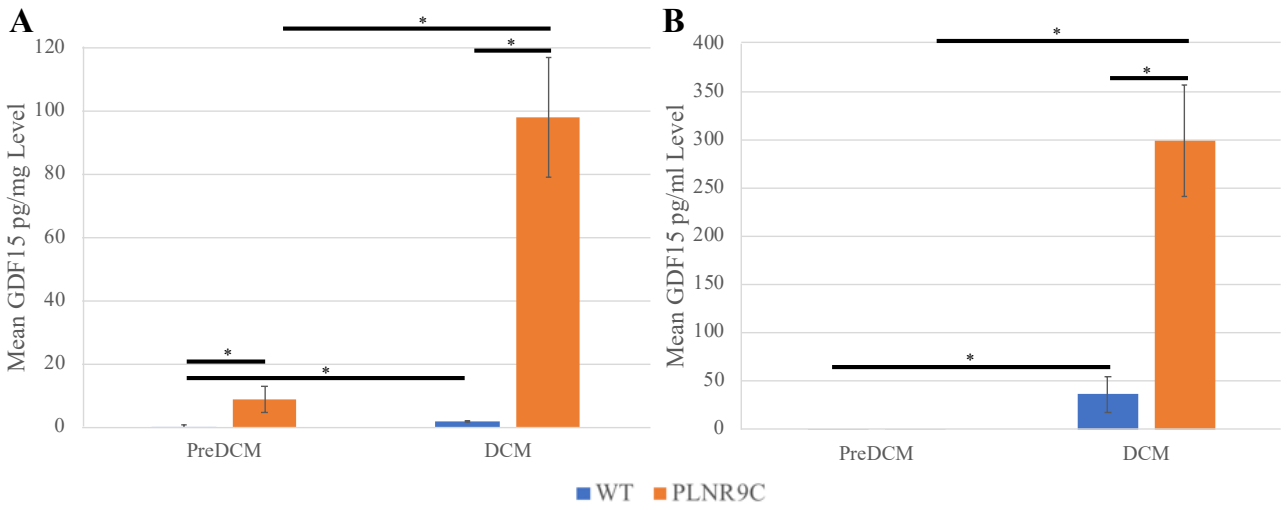


Figure 8. ***GDF15 is upregulated in PLN^{R9C} hearts.*** **(A)** Progressive and marked increase was observed in GDF15 protein levels in LV tissue in PLN$^{R9C/+}$ mice (*p<0.05). **(B)** Progressive and substantial increase was observed in circulating GDF15 protein level in serum (*p<0.05). N=4 mice/genotype; *t* test.

GDF15 alters body composition in PLN$^{R9C/+}$ mice.

To understand the role of GDF15 in cardiac cachexia, we measured fat and lean muscle mass with the onset of overt HF symptoms in PLN$^{R9C/+}$ mice with or without GDF15, or age/sex-matched WT and *Gdf15* KO mice. *Pln$^{R9C/+}$-Gdf15$^{+/+}$*displayed severe wasting of white (inguinal, epididymal, retroperitoneal) and brown (interscapular) fat with progression to overt HF (p<0.001 for all comparisons, n=19/genotype; Fig. 9A). Similarly, *Pln$^{R9C/+}$-Gdf15$^{+/+}$*mice developed sarcopenia with significant reductions in muscle mass compared to WT (p<0.001 for gastrocnemius and p=0.001 for tibialis, n=19/genotype; Fig. 9B). With GDF15 KO in PLN$^{R9C/+}$ mice, fat and muscle mass were preserved relative to *Pln$^{R9C/+}$-Gdf15$^{+/+}$*mice (p<0.001 for all fat comparisons, p<0.001 for gastrocnemius, and p=0.014 for tibialis, n=19/genotype).

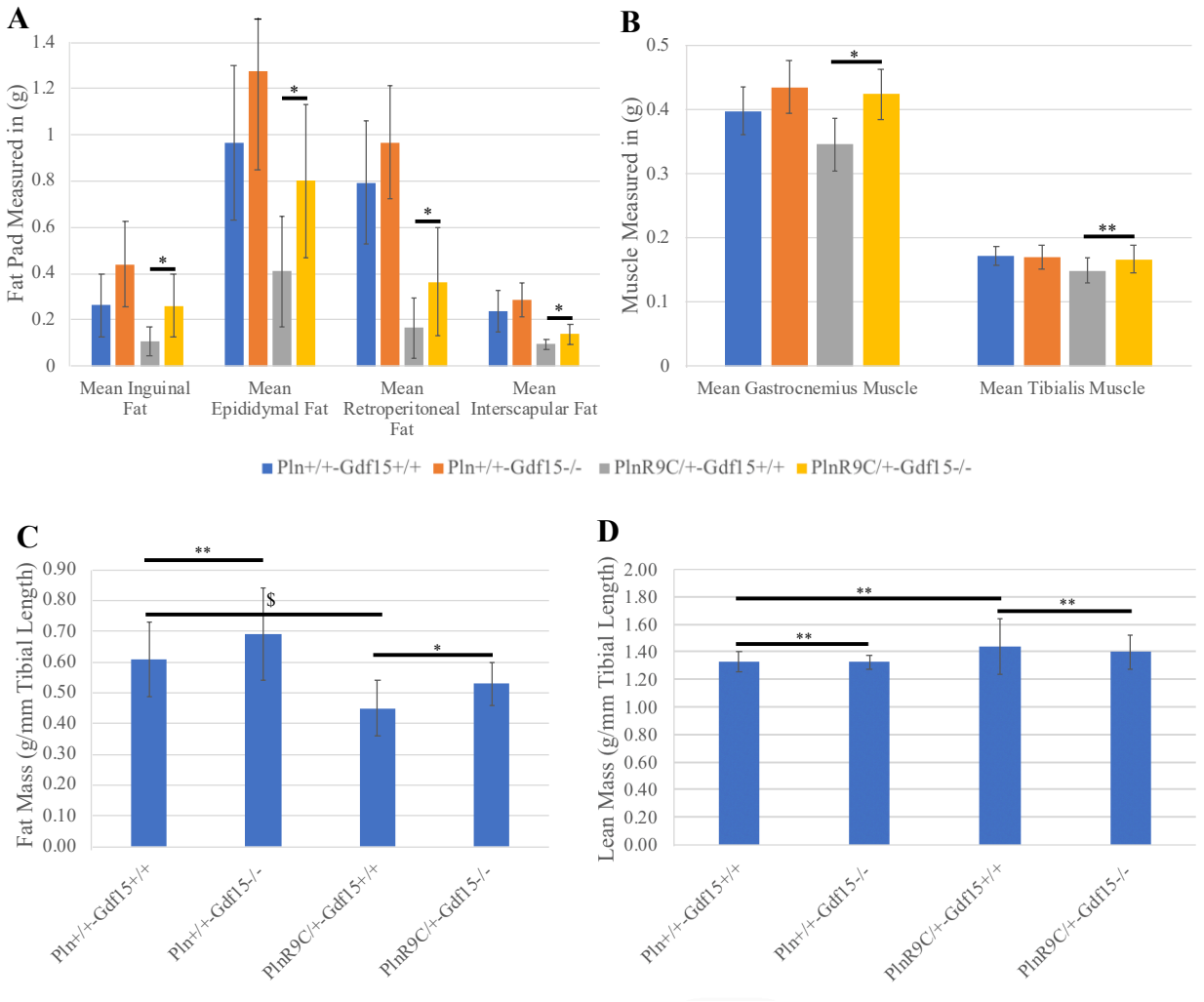


Figure 9. GDF15 alters body composition in PLN$^{R9C/+}$ mice. (A) Preserved fat mass was seen in *Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$ mice (*p<0.001). **(B)** Preserved muscle mass was seen in *Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$ mice (**p=0.014). **(C)** DXA scan showed decreased fat mass in *Pln*^{R9C}-*Gdf15*$^{+/+}$ mice (*p=0.039 vs. *Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$ & $p=0.010 vs. WT). **(D)** DXA scan showed no difference in lean mass in *Pln*^{R9C}-*Gdf15*$^{+/+}$ vs. WT & *Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$ (**p=n.s. for all DXA analyses). N=4 mice/genotype; *t* test.

We also performed DXA scanning to assess body composition. There was no significant difference in fat mass in WT mice by GDF15 KO status (0.61 vs. 0.69 g/mm, p=0.19, n=9/*Pln*$^{+/+}$-*Gdf15*$^{+/+}$ and n=14/*Pln*$^{+/+}$-*Gdf15*$^{-/-}$; Fig. 9C). Similarly, lean mass remained unchanged in WT mice, demonstrating that GDF15 does not affect body composition at baseline (1.33 vs 1.33 g/mm, p=0.97; Fig. 9D). By contrast, *Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$mice displayed a ~ 30% reduction in fat mass

compared to WT (0.45 vs. 0.69 g/mm, p=0.01, n=10/*Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$; Fig. 9C). *Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$ mice again showed preserved fat mass relative to *Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$ (0.53 vs. 0.45 g/mm, p=0.04, n=17/*Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$). *Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$mice did not have lower lean mass by DXA, in contrast to our tissue analysis (1.44 vs. 1.33 g/mm *Pln*$^{+/+}$-*Gdf15*$^{+/+}$, p=0.17; 1.44 vs. 1.40 g/mm *Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$, p=0.488; Fig. 9D). This is likely secondary to variable but significant amounts of edema that these mice develop as DCM progresses and which cannot be readily separated from lean tissue mass by this technique. Collectively, these data demonstrate that GDF15 is associated with the development of cachexia in PLN$^{R9C/+}$ mice with progression of DCM to HF.

GDF15 reduces food intake.

To better characterize the nature of the weight loss in PLN$^{R9C/+}$ mice, we measured food intake prior to the onset of HF in mice with or without GDF15 KO (Fig. 10). Food intake was virtually identical between WT cohorts (5.1 ± 0.6 g/week; p=0.995, n=10/genotype). *Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$ mice ate less food than age/sex-matched WT (3.5 ± 0.5 g/week; p<0.001, n=10/genotype). By contrast, *Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$ ate significantly more food relative to *Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$ (4.0 ± 0.5 g/week; p=0.027, n=10/genotype), though it is noted they still consumed less food than WT mice (p<0.001). Thus, GDF15-induced cachexia may be at least in part related to hypophagia/anorexia.

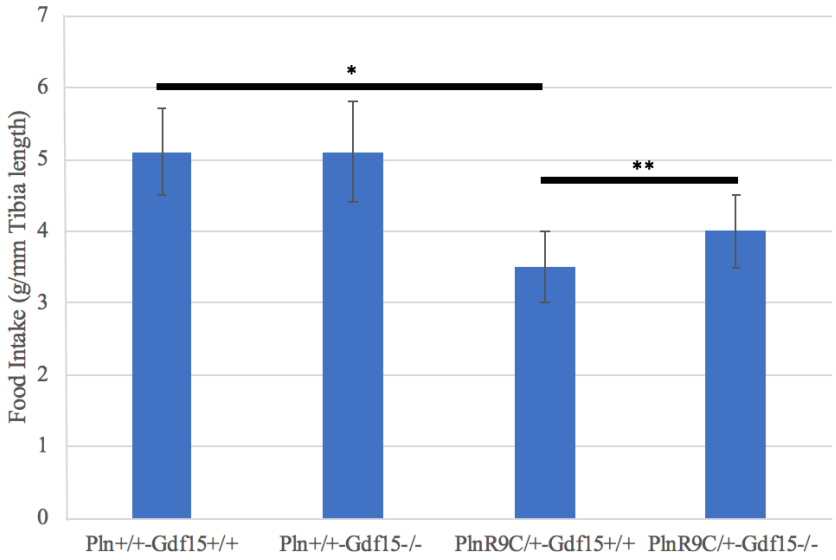


Figure 10. GDF15 reduces food intake. Significant decrease in food intake was observed in $Pln^{R9C/+}$-$Gdf15^{+/+}$mice (*p<0.001 vs. WT). Significant increase in food intake was observed in $Pln^{R9C/+}$-$Gdf15^{-/-}$ mice (**p=0.027 vs. $Pln^{R9C/+}$-$Gdf15^{+/+}$). N=10 mice/genotype; *t* test.

GDF15 KO increases survival.

To ascertain whether GDF15 was associated with survival, $Pln^{R9C/+}$-$Gdf15^{+/+}$ and $Pln^{R9C/+}$-$Gdf15^{-/-}$ mice were followed longitudinally for survival (Fig. 11). Lifespan was 15% longer in $Pln^{R9C/+}$-$Gdf15^{-/-}$ mice (29 ± 3 weeks vs. 25 ± 3 weeks; log-rank, p<0.001, n=32 mice/$Pln^{R9C/+}$-$Gdf15^{+/+}$, n=39 mice/$Pln^{R9C/+}$-$Gdf15^{-/-}$).

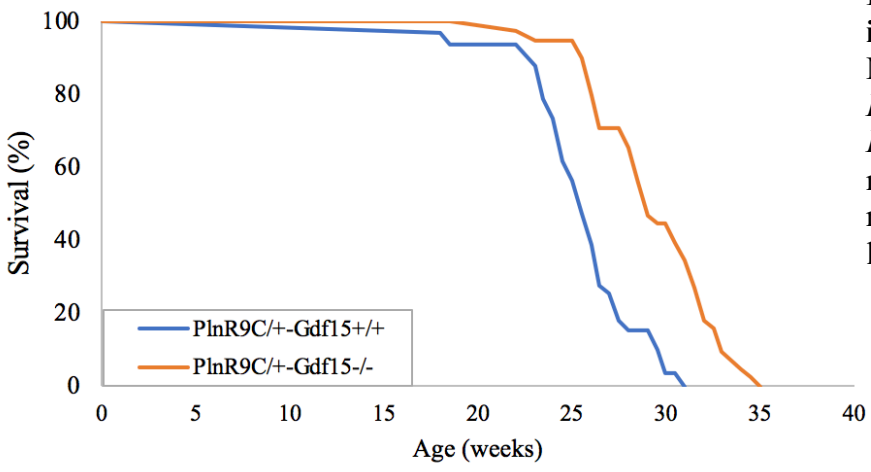


Figure 11. GDF15 KO increases survival. Kaplan-Meier plot of survival in $Pln^{R9C/+}$-$Gdf15^{+/+}$ and $Pln^{R9C/+}$-$Gdf15^{-/-}$ mice (n=32 mice/$Pln^{R9C/+}$-$Gdf15^{+/+}$; n=39 mice/$Pln^{R9C/+}$-$Gdf15^{-/-}$, log-rank test).

GDF15 does not affect cardiac structure or function in chronic HF.

To assess whether GDF15 affects cardiac structure and/or function in $PLN^{R9C/+}$ mice, we performed echocardiography (cardiac ultrasound) longitudinally from 10-22-weeks of age (Fig. 12). *Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$mice developed severe, progressive DCM as evidenced by progressive LV dilation, wall thinning and reduced LV systolic function compared to WT mice (LVEDD: 4.5 ± 0.4 mm vs. 3.2 ± 0.3 mm; LVWT: 1.3 ± 0.2 mm vs. 1.6 ± 0.2 mm; FS: 17% ± 5% vs. 45% ± 7%; LVESD: 3.8 ± 0.6 mm vs. 1.8 ± 0.3 mm; n=10 *Pln*$^{+/+}$-*Gdf15*$^{+/+}$, n=17 *Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$; p<0.001 for all). GDF15 KO had no apparent effect relative to *Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$ (LVEDD: 4.2 ± 0.5 mm vs. 4.5 ± 0.4 mm, p=0.22; LVWT: 1.3 ± 0.3 mm vs. 1.3 ± 0.2 mm, p=0.71; FS: 22 % ± 7% vs. 16% ± 6%, p=0.48; LVESD: 3.4 ± 0.6 mm vs. 3.8 ± 0.6 mm, p=0.38; n=17 *Pln*^{R9C}-*Gdf15*$^{+/+}$, n=18 *Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$).

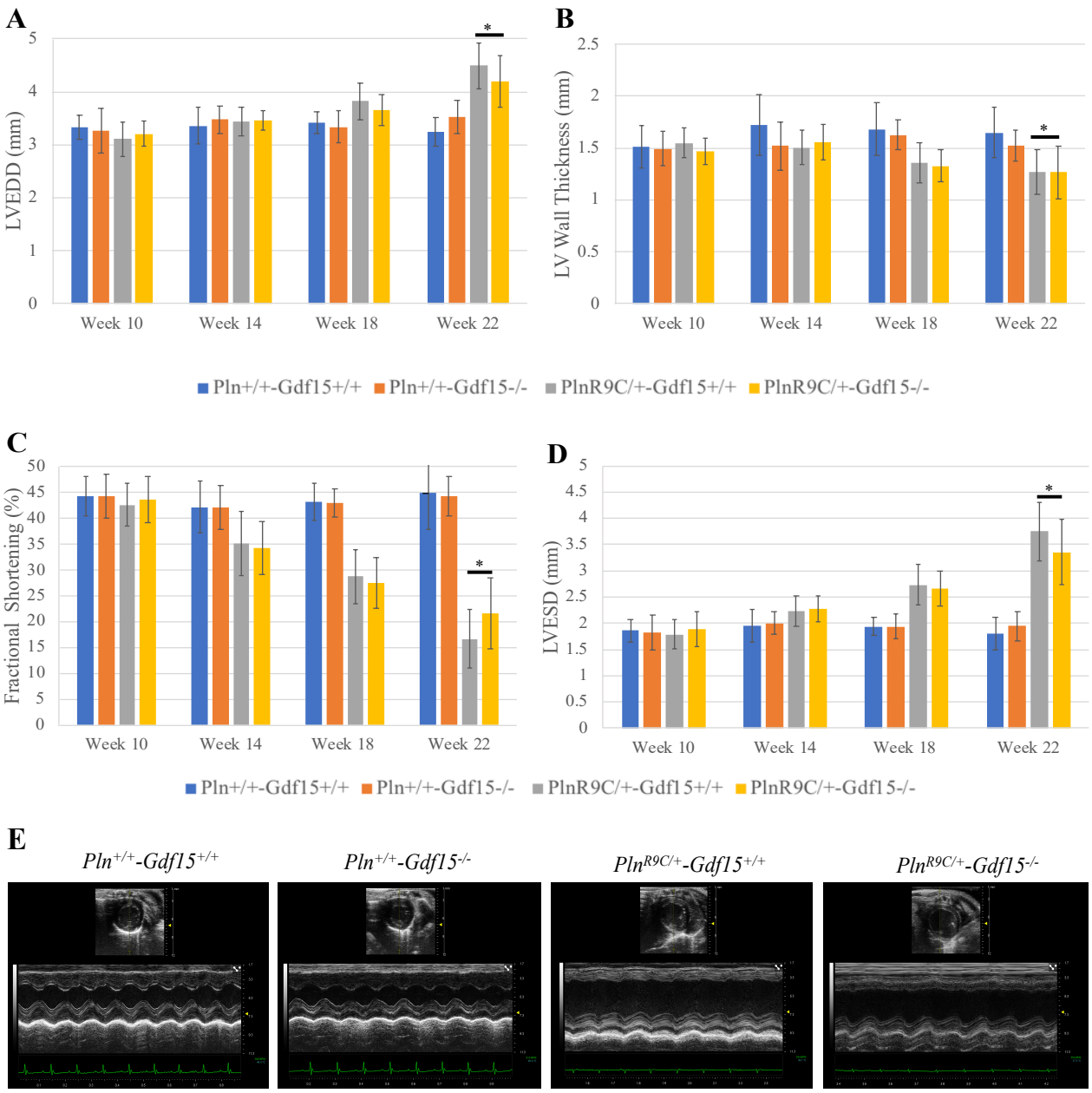


Figure 12. GDF15 does not affect cardiac structure or function in chronic HF.
No difference in cardiac structure was seen as measured by **(A)** LV end diastolic diameter (LVEDD) or **(B)** LV wall thickness in PLN$^{R9C/+}$ mice by GDF15 KO status. Similarly, no difference in cardiac function was seen as measured by **(C)** fractional shortening or **(D)** LV end systolic diameter (LVESD) in PLN$^{R9C/+}$ mice by GDF15 KO status. **(E)** Representative echocardiographic M-mode images. (*p=n.s. for all analyses, n=10/*Pln*$^{+/+}$-*Gdf15*$^{+/+}$, n=11/*Pln*$^{+/+}$-*Gdf15*$^{-/-}$; n=17/*Pln*$^{R9C/+}$-*Gdf15*$^{+/+}$, n=18/*Pln*$^{R9C/+}$-*Gdf15*$^{-/-}$; ANOVA).

We assessed cardiac fibrosis using picrosirius red stained LV tissue sections from 22-week old mice (Fig. 13A). WT mice had no significant myocardial fibrosis (1.4% vs. 1.1%, p=0.24; n=3/genotype; Fig. 13B). Consistent with our lab's prior publications, $Pln^{R9C/+}$-$Gdf15^{+/+}$ mice developed severe cardiac fibrosis (21.3% vs. 1.4% $Pln^{+/+}$-$Gdf15^{+/+}$; 15.2% relative increase; p<0.001; n=3/genotype). $Pln^{R9C/+}$-$Gdf15^{-/-}$ mice had 16% less total cardiac fibrosis than $Pln^{R9C/+}$-$Gdf15^{+/+}$ (18.2% vs. 21.3% , p=0.001). However, the absolute reduction in fibrosis was small (net change 3.1%), thus raising the question of whether this difference was biologically significant.

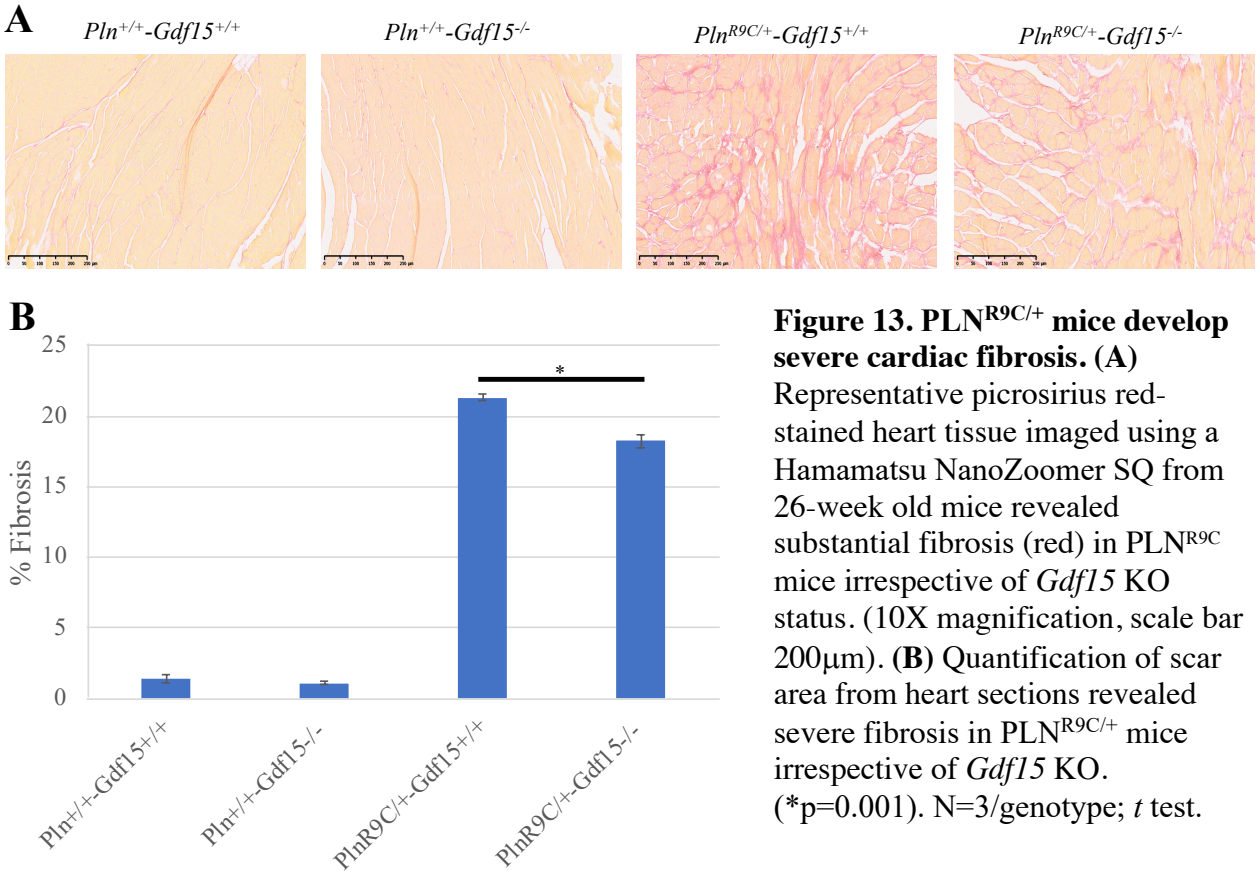


Figure 13. $PLN^{R9C/+}$ mice develop severe cardiac fibrosis. (A) Representative picrosirius red-stained heart tissue imaged using a Hamamatsu NanoZoomer SQ from 26-week old mice revealed substantial fibrosis (red) in PLN^{R9C} mice irrespective of *Gdf15* KO status. (10X magnification, scale bar 200µm). **(B)** Quantification of scar area from heart sections revealed severe fibrosis in $PLN^{R9C/+}$ mice irrespective of *Gdf15* KO. (*p=0.001). N=3/genotype; *t* test.

DISCUSSION

Cardiac cachexia is a morbid complication in HF. Herein, I have identified GDF15 as a putative causal cardiac-specific molecular signal that triggers cardiac cachexia in HF. By parsing gene expression into different types of organs (lung, spleen, brain, kidney, liver, muscle, fat, small intestine, colon, and heart), I confirmed that the previously identified upregulation of GDF15 is cardiac specific and correlates with increased circulating levels in this chronic model of HF. By comparison of multiple metrics of cachexia (tissue weight, body composition, food intake), we discovered that *Gdf15* KO reduces cardiac cachexia. Interestingly, this reduction in cachexia coincided with a slight but highly significant prolongation in survival. By contrast, we found that *Gdf15* KO does not substantially alter cardiac structure and function, suggesting that the primary effect of GDF15 is systemic, and thereby confirming GDF15 as a novel cardiac hormone. This also identifies GDF15 as a potential novel therapeutic target for patients with chronic HF.

Patients with cancer and HF are both prone to developing significant cachexia, particularly in late stages of the disease.[18] Though it has long been believed this is due to a multifactorial process involving a complex interplay between different systems, recent evidence in the cancer field has identified GDF15 as a central, tumor-derived activator of cachexia.[19] Animal studies have revealed this effect to be due at least in part to anorexia produced by systemic release of GDF15 in a variety of model systems.[19,22-24] Herein we have established that GDF15 is a key mediator of cardiac cachexia in a mouse model of chronic HF. We confirmed our results using 2 separate experimental strategies (direct tissue weight and live-mouse imaging with DXA). Though we did not find a reduction in lean body mass via DXA scanning, it is noted that excess total body water cannot be separated from lean tissue mass by DXA; hence, lean mass is artifactually high due to the edema these mice developed with the onset of HF. Finally, our KO mouse model revealed that

GDF15 also controls food intake, suggesting anorexia as the mechanism by which GDF15 causes cardiac cachexia.

Collectively, these data identify GDF15 as a novel cardiac hormone that regulates food intake and drives cardiac cachexia. Presently, the only known cardiac hormones are BNP (brain-type natriuretic peptide) and ANP (atrial natriuretic peptide).[72] Thus the paucity of cardiac hormones makes our finding highly important as it identifies a new cardiac endocrine function, and suggests that more may await future discovery.

In contrast to prior studies, we found no change in cardiac structure or function with GDF15 KO in HF. While previous studies have found GDF15 to be cardioprotective in models of MI[59,60] and pressure overload[61], an intracardiac mechanism for these findings remains unclear. In MI, the mechanism of cardioprotection was extracardiac via regulation of the systemic inflammatory response that characterizes acute MI; no intracardiac signaling mechanism was identified.[60] In TAC, the authors implicated the SMAD3 signaling pathway as the putative mechanism for GDF15 to reduce cardiac hypertrophy.[61] SMAD3 activation is a hallmark feature of TGFβ signaling and GDF15 is a distantly related member of the TGFβ superfamily, leading to a plausible but ultimately false narrative as a later study found that the recombinant GDF15 preparations used in this study were contaminated with a measurable amount of TGFβ, thus possibly invalidating these findings.[73]

Notably, both the MI and TAC models are models of acute HF and both are reasonably artificial models. In MI, adolescent or young adult mice are almost invariably studied, while in humans, this demographic virtually never suffers MI except in the extremely rare setting of homozygous familial hypercholesterolemia. TAC is a model of pressure overload such as that seen in hypertension (the commonest medical diagnosis in the world and the most common risk factor for development of HF, estimated to be causative in at least 20% of HF patients)[5] or aortic stenosis

(the commonest valve disease in Western countries). However, unlike in humans where these conditions almost exclusively develop in later life and where the disease (and therefore the cardiac stress it causes) develops slowly over decades, in TAC pressure overload is induced acutely in adolescent or young adult life, producing a cardiac remodeling environment unlike that seen in the vast majority of human HF. Therefore, a particular strength of our study is the use of the PLN^{R9C} mouse model, which closely mimics human disease and mirrors a common molecular process (deranged calcium handling) that is found in nearly all forms of HF studied. Hence, our model represents a chronic model of HF while TAC is a model of acute HF dissimilar to most forms of human HF. Thus, our contrasting results suggest that the effects of GDF15 may be distinct in acute and chronic HF, and leave unanswered the mechanism of cardioprotection in acute disease.

Despite the lack of apparent effect on cardiac remodeling, we identified a survival benefit in HF mice with *Gdf15* KO. As noted, cachexia in HF identifies a particularly sick cohort with exceedingly high mortality.[12-15] Hence, a survival benefit observed in the absence of an effect on cardiac remodeling points to an extra-cardiac mechanism. It is tantalizing to suggest that the survival benefit is due to a reduction in cardiac cachexia, though additional data are needed to support this hypothesis.

Our study has several limitations. Though we have confirmed GDF15 as a novel cardiac hormone that induces cachexia, we have not identified the mechanism of action. The GDF15-GFRAL axis is a clearly established pathway for regulating food intake, marking this as a key area of future investigations for our lab. However, recent evidence has also suggested non-central mechanisms of GDF15 regulation on fat metabolism.[74] Further investigations are needed to assess if a peripheral mechanism is active in our model. Finally, while we postulate the difference in lean tissue findings between our tissue weight and DXA studies is due to excess peripheral edema in

$PLN^{R9C/+}$ mice, we cannot rule out that sarcopenia may actually be minimal and additional studies on skeletal muscle to assess for GDF15-induced differences are warranted. I plan to perform immunohistochemistry on hindbrain tissue sections to assess for GFRAL activation. I will also perform RNA-seq on fat tissues to begin to assess the changes in gene expression that could be triggering fat tissue loss in HF mice relative to those with *Gdf15* KO. Finally, we will create a 2nd transgenic model by crossing $PLN^{R9C/+}$ to a *Gfral* knockout mouse (Taconic #TF3754). This mouse will also allow us to decisively define the role of the GDF15-GFRAL axis in cardiac cachexia.

Cardiac cachexia is a common and highly morbid complication of HF. Despite this, there are no therapeutic strategies that target cardiac cachexia and, to our knowledge, none are in development. We provide the first evidence for a novel cardiac-specific molecular mechanism that drives cachexia in HF. We have identified GDF15 as a new cardiac hormone that regulates cardiac cachexia and improves survival in a mouse model of chronic, progressive HF. Targeting the GDF15 pathway has recently become a very intense area of research in the pharmaceutical industry. The goal of this research is to develop novel treatments for obesity and anorexia/cachexia syndromes. Hence, our research could readily translate into a novel therapeutic strategy to treat cachexia in patients with HF, a first in class strategy that would offer the first hope for those suffering with this devastating and fatal complication.

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