Hematoxylin & Eosin (H&E) and Immunohistochemistry (IHC)

Paraffin embedded sections were incubated with antibodies: desmin (Abcam #ab15200, 1:200), chymase (Abcam #ab2377, 1:50) and Alexa Fluor 488- and 594-conjugated secondary antibodies (1:700, Life Technologies) and appropriate host combinations were used to stain for visualization. Nuclei were labeled with DAPI (1.5µg/ml, Vector Laboratories #H-1500).

Collagen Analysis

Collagen quantification was performed on MR and non-failing atrial tissue immersion-fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4µm thickness, and stained with Picric Acid Sirius Red F3BA. Quantitative analysis was performed by light microscopy (Leica Microsystems, Bannockburn, IL) with a Hamamatsu ORCA ER cooled CCD camera and a digital-based image-analyzer system (Image-Pro Plus, MediaCybernetics, San Diego, CA). Collagen volume percent was quantitatively evaluated at low power (10x objective, 800x total magnification) for total collagen. The Picric Acid Sirius-stained sections were viewed with a 540-nm (green) filter to provide grayscale contrast of the collagen with the background. Volume percent collagen was determined from 10 to 20 randomly selected fields in each section, and the mean value was calculated for each patient. All morphometric measurements were performed in a blinded manner. Results are presented as the mean ± SD values computed from the average of individual measurements obtained from each biopsy.

Western blots for Myosin

Tissue (RA, LA or LV) was powdered with a mortar & pestle under liquid nitrogen then homogenized in RIPA buffer containing protease and phosphatase inhibitors (PIERCE). Tissue homogenates (2.5ug) were denatured at 95°C for 5 minutes in NuPAGE™ LDS Sample Buffer containing NuPAGE™ sample reducing agent (Invitrogen). Samples were separated on a NuPAGE™ Novex 6% Tris-Glycine protein gels (Invitrogen), transferred to a 0.2µm PVDF membrane (Invitrogen) and probed with antibodies to myosin heavy chain MF-20 (mouse

monoclonal; 1:400; DHSB) or GAPDH (rat; 1:2500; Abcam #ab9485) for 4 hours at room temperature in 5% Blotto in phosphate buffered saline + 0.1% Tween-20 (PBST). Membranes were washed in PBST then incubated in horse radish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad Laboratories; 1:5000) for 1-1.5 hours at room temperature in 5% Blotto. HRP signals were developed using Clarity Western enhanced chemiluminescence substrate (Bio-Rad Laboratories) on a FluorChem M system (Protein Simple).

Transmission electron microscopy (TEM)

Ten MR biopsies were fixed in 2.5% Glutaraldehyde/Sorensen's Phosphate Buffer (Electron Microscopy Sciences #15980) overnight at 4°C. Tissue was processed for TEM and sections were viewed in a Philips 201 TEM (FEI Co.) by EMLabs Inc (Birmingham, AL) for qualitative changes in ultrastructure. For immunogold TEM, human heart biopsies were submerged in 0.1% glutaraldehyde/2% paraformaldehyde in cardioplegic buffer (5% dextrose, 30 mmol/L KCl in PBS), further fixed in the same fixative in cacodylate buffer, incubated in 0.1 mol/L glycine/PBS, dehydrated in series of N, N-dimethyl formamide, and embedded in LRWhite resin. Ultrathin (90 nm) sections (10-12) were picked up on nickel grids (3 mm) and dried. Before immunogold labeling, grids were rinsed in PBS and blocked for 1.5 h with 1% BSA, 0.1% cold water fish skin gelatin, and 1% Tween 20 in PBS. Grids were then incubated with chymase antibody (Bioss 2353R), diluted 1:25 with 1% BSA/PBS first for 2 h at room temperature (RT) and then overnight at 4°C. The grids were rinsed with PBS, blocked and incubated (RT, 2h) with goat anti-rabbit IgG tagged with colloidal gold (10-nm particle size, diluted 1:50 with 1% BSA/PBS) (Aurion/Electron Microscopy Sciences). Sections were postfixed and counterstained with uranyl acetate and viewed on a FEI-Tecnai T12 Spirit 20 electron microscope.

Chymotryptic Assay

A modified extraction method was used to isolate tissue chymase from RA, LA or LV using a two buffer extraction method as previously described in our laboratory.[13] Briefly, tissue (10-20 mg) was homogenized in ice cold low salt lysis buffer (10mM Tris-Cl, pH 8.0, 1 mM

EDTA containing the following inhibitors: E-64, Pepstatin A, Bestatin hydrochloride, 4-chloromercuribenzoic acid, Lisinopril all at 50μM, amastatin hydrochloride at 10 μM, and epoxomicin at 1 μM), and subjected to a freeze/thaw cycle five times to break up cells and tissue compartments, centrifuged at 16,000 x *g* for 30 minutes and the supernatant discarded. The pellet was re-suspended in 1 mL of ice cold low salt buffer and incubated on a rotator for 3 hours at 4°C, followed by centrifugation at 10,000 x *g* for 30 minutes and the supernatant set aside (low salt extract). The pellet was re-suspended in high salt lysis buffer (100mM Tris-Cl, pH 8.0, 1mM EDTA, 2M NaCl containing the same inhibitors listed above for the low salt buffer) overnight with rotation at 4°C to extract chymase. Samples were centrifuged at 10,000 x *g* for 30 minutes and the supernatant collected for enzymatic assay (high salt tissue extract). Chymotryptic enzyme activity was measured in 0.2 mL of high salt tissue extract with the addition of the fluorescent substrate N-Succinyl-Ala-Ala-Pro-Phe-7-amido-4 methylcoumarin (10μM, Sigma S9761). All samples and fluorescent standards of the chromophore 7-Amino-4-methylcoumarin (Sigma A9891) were run in duplicate on a SpectraMax i3x (Molecular Devices) in a 96-well kinetic plate assay for 1 hour (1 minute interval reads) at 37°C with an excitation spectrum of 360nm and emission of 460nm. Protein concentration in high salt extracts was quantified with the CB Protein Assay kit (G Biosciences 786-012) with bovine serum albumin (BSA) as the standard. Chymotryptic activity was expressed as fmol/mg/min. The chymotrytic activity is only found in the high salt extract.[13]

In situ chymotryptic activity

Chymase activity was verified by *in situ* chymotryptic activity as described previously by our laboratory.[13] Briefly, fresh LV tissue was fixed in 4% paraformaldehyde for 6 hours at room temperature, transferred to 25% sucrose overnight at 4°C, then embedded in OCT compound (Tissue-Tek #4583). Fixed-frozen 5μm sections were thawed, washed 3 times in PBS, and incubated in Reaction Mixture at 37°C for 3 hours. Sections were rinsed in water and counterstained with Basic Fuchsin (Electron Microscopy Sciences #11260, 0.05%) for 1-2

seconds to achieve a pale pink background, and viewed under bright field microscopy. The Reaction Mixture consisted of 0.53mM Napthol AS-D chloroacetate (MP Biomedical #02102424.1), 0.3mM Fast Blue BB Salt (Sigma #44670) dissolved in N,N-Dimethylformamide (Sigma #D4451, final 3.8%) vortexed and then immediately added to a final concentration of 50mM Tris-Cl pH 7.5 (Fisher #BP154-1), 40mM NaF (Acros Organics #191270250), and 1% Triton X-100 (Fisher #BP151). The mixture was stirred for 2-3 minutes until a dark blue color appeared. The solution was prepared in the dark, filtered and used immediately.

Chymase mRNA production by in situ hybridization

The expression of human chymase (CMA1) mRNA was measured by *in situ* hybridization as previously described in our laboratory.[13] The CMA1 cDNA flanked by the KpnI and EcoRI restriction sites (sequence shown below) was synthesized and cloned to the pBluescript II KS(+) vector by GenScript (Piscataway, NJ, USA). The resulting construct was confirmed by sequencing. The antisense and sense probes were synthesized with a digoxigenin (DIG) RNA labeling kit (Roche**,** #11175025910) utilizing the T7 and T3 RNA polymerase promoter in the pBluescript II KS(+) vector, respectively. *In situ* hybridization was carried out on 5μm formalin-fixed, paraffin embedded atrial sections from MR patients using the antisense CMA1 probe. The sense probe was used as a control.

ggtaccgccacc atgctgcttc ttcctctccc cctgctgctc tttctcttgt gctccagagc tgaagctggg gagatcatcg ggggcacaga atgcaagcca cattcccgcc cctacatggc ctacctggaa attgtaactt ccaacggtcc ctcaaaattt tgtggtggtt tccttataag acggaacttt gtgctgacgg ctgctcattg tgcaggaagg tctataacag tcacccttgg agcccataac ataacagagg aagaagacac atggcagaag cttgaggtta taaagcaatt ccgtcatcca aaatataaca cttctactct tcaccacgat atcatgttac taaagttgaa ggagaaagcc agcctgaccc tggctgtggg gacactcccc ttcccatccc aattcaactt tgtcccacct gggagaatgt gccgggtggc tggctgggga agaacaggtg tgttgaagcc gggctcagac actctgcaag aggtgaagct gagactcatg gatccccagg cctgcagcca cttcagagac tttgaccaca atcttcagct gtgtgtgggc aatcccagga agacaaaatc tgcatttaag ggagactctg ggggccctct tctgtgtgct ggggtggccc agggcatcgt atcctatgga cggtcggatg caaagccccc tgctgtcttc acccgaatct cccattaccg gccctggatc aaccagatcc tgcaggcaaa ttggaattc

Table 1. Mitral Regurgitation Demographics and Baseline Echo Data. — indicates transesophageal echo/Doppler only.											
Patient ID	**Age (yrs)**	**Sex**	**Race**	**Medications**	**HBP**	**AF (Pre)**	**NYHA**	**LVEDD (cm)**	**LVESD (cm)**	**LVEF (%)**	**LAD (cm)**
1	60	Female	White	Atenolol, flecainide, rivaroxaban	No	Yes	3	5.4	3.4	65	—
2	82	Male	White	Apixaban, furosemide, lisinopril	No	No	3	4.6	3.4	50	5.9
3	62	Male	White	Glucophage, HCTZ	No	Yes	1	5.3	3.2	60	3.0
4	50	Male	White	None	Yes	No	2	5.6	3.7	63	4.2
5	48	Female	Black	None	No	No	2	—	—	55	—
6	48	Male	White	None	No	No	3	5.6	3.5	67	5.6
7	68	Female	White	Furosemide, metoprolol, losartan	No	No	3	—	—	65	—
8	73	Male	White	Pravastatin	No	No	1	4.6	3.1	60	3.9
9	73	Male	White	None	Yes	No	1	6.6	4.4	72	5.7
10	53	Female	White	None	No	No	3	4.6	3.9		4.9
11	77	Male	White	Rivaroxaban, diltiazem, rosuvastatin	No	Yes	2	—	—	55	—
12	63	Male	Black	Carvedilol, furosemide, lisinopril, metformin, statin	Yes	No	2	—	—	60	—
13	58	Male	White	Metoprolol, statin		No	2	5.1	3.5	60	3.1
14	39	Male	White	None	No	No	3	6.3	3.5	72	6.6
15	55	Female	Black	Lisinopril, statin, glyburide	Yes	No	3	4.9	2.7	70	—
16	56	Female	Black	Atenolol	Yes	No	2	4.5	2.6	70	3.1
17	50	Male	White	Metoprolol, apixaban, flecainide	Yes	Yes	2	4.8	3.5	60	
18	71	Male	White	None	Yes	No	2	5.1	3.5	65	3.1
19	47	Male	White	Lisinopril, metoprolol	Yes	No	1	6.0	3.8	60	5.1
20	64	Male	White	Hydralazine	Yes	No	2	5.1	2.1	80	5.3

Table 2. Patient Demographics of Non-Failing Heart Specimens.								
Patient ID	**Age**	**Sex**	**Race**	**BMI**	**CAD**	**HBP**	**LVH**	**LVEF**
1	38	Male	White	35	Yes	No	No	65
2	54	Male	Black	17	No	No	No	50
3	67	Male	Black	23	Yes	Yes	No	60
4	42	Female	White	23	No	No	No	57
5	56	Female	White	28	Yes		Yes	60
6	59	Male	White	25	No	No	No	65
7	51	Male	White	39	No	Yes	No	55
8	62	Male	Black	28	No	Yes	Yes	55
9	59	Male	White	27	No	Yes	No	65
10	33	Female	White		No	Yes	Yes	65
11	36	Male	White	22	No	No	No	55
12	55	Male	Black	20	No	Yes	No	70
13	59	Female	White	20	Yes	Yes	No	60
14	51	Female	White	24	No	No	No	70
15	30	Female	Black	21	No	No	No	60

Table 3. Baseline MRI volumes Regurgitant Volume and Regurgitant Fraction	
MRI volumes at Baseline	**Summary measure**
Number of patients	20
LVEDV (ml)	185 (160, 223)
LVESV (ml)	67 (57, 101)
LVSV (ml)	116 (97, 140)
LVEF (%)	60 (56, 66)
LV Regurgitant Volume (ml)	54 (22, 67)
Regurgitant fraction (%) [a]	44% (23%, 56%)

Data presented as median interquartile range (Q1, Q3), ml= milliliters
Regurgitant fraction calculated by dividing LV regurgitant volume/LV stroke volume
MRI: Magnetic resonance imaging

Supplemental Figure 1

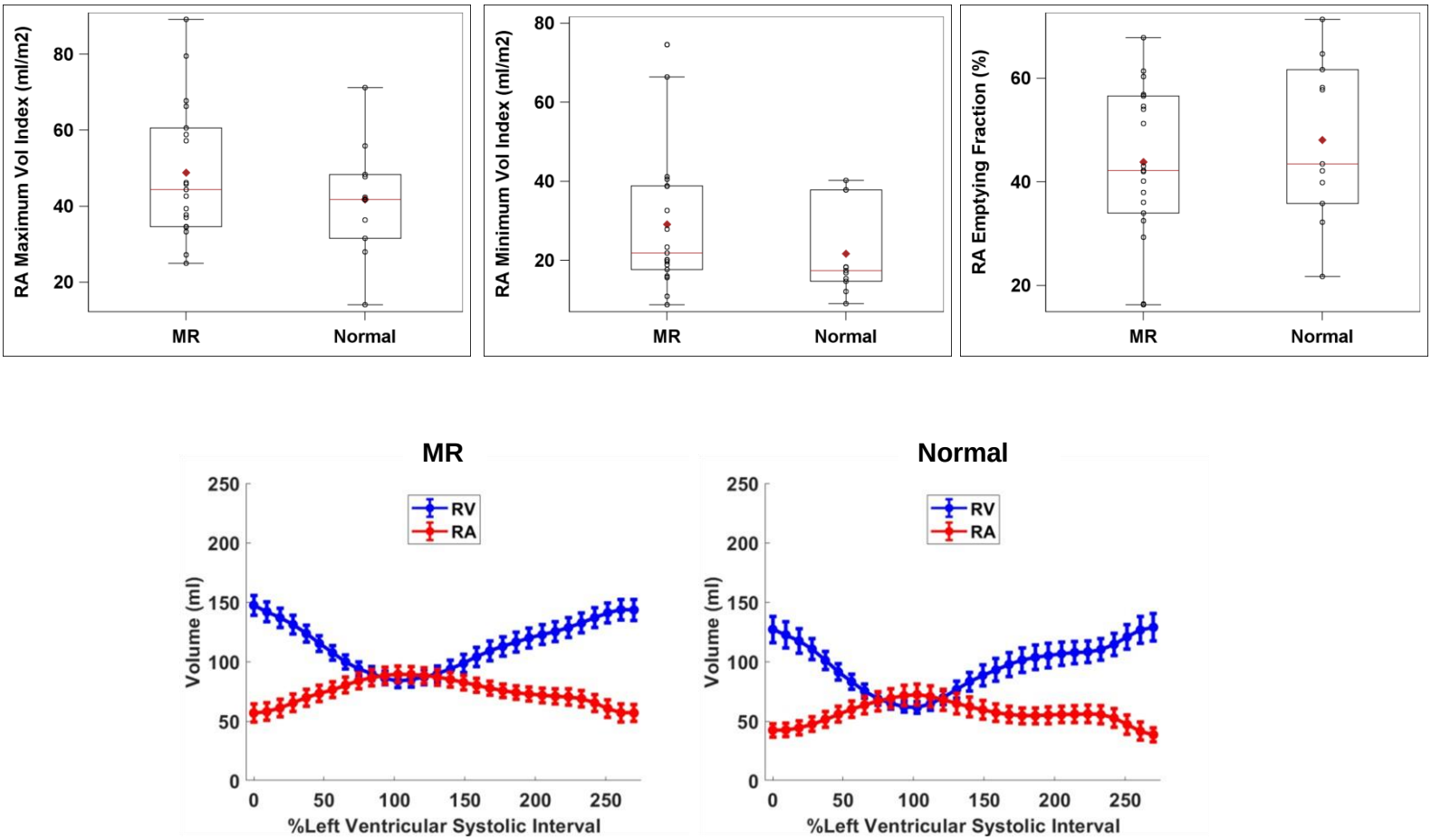
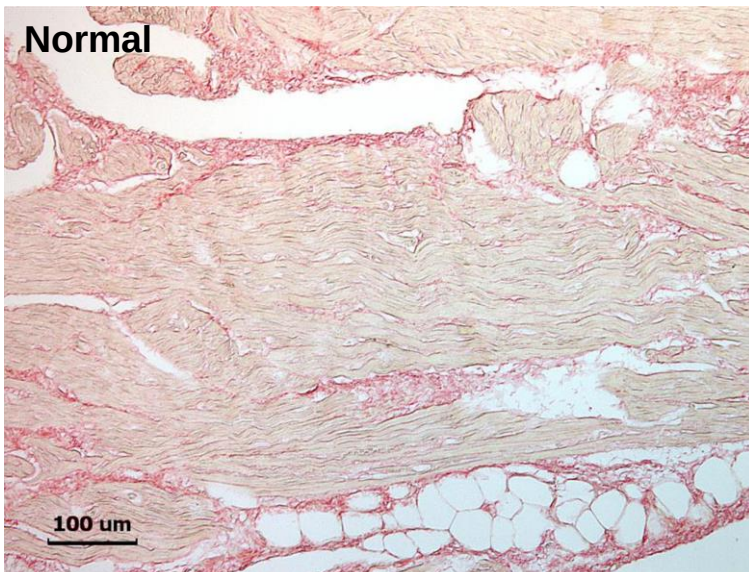
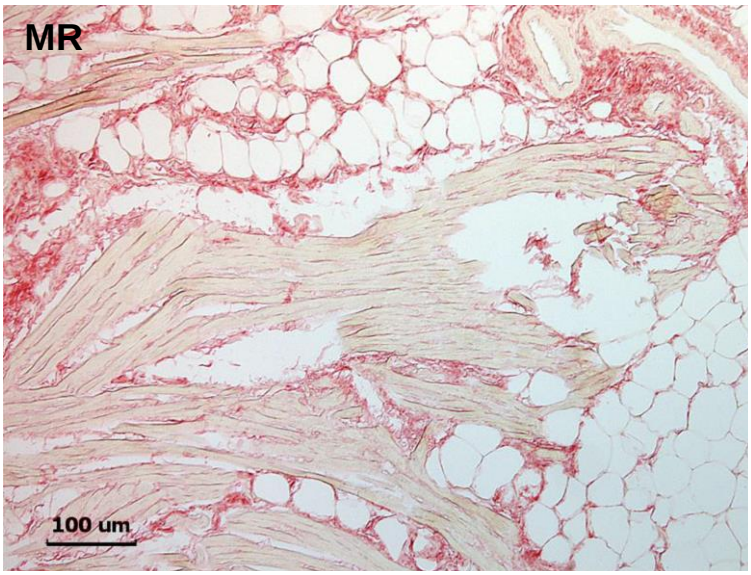
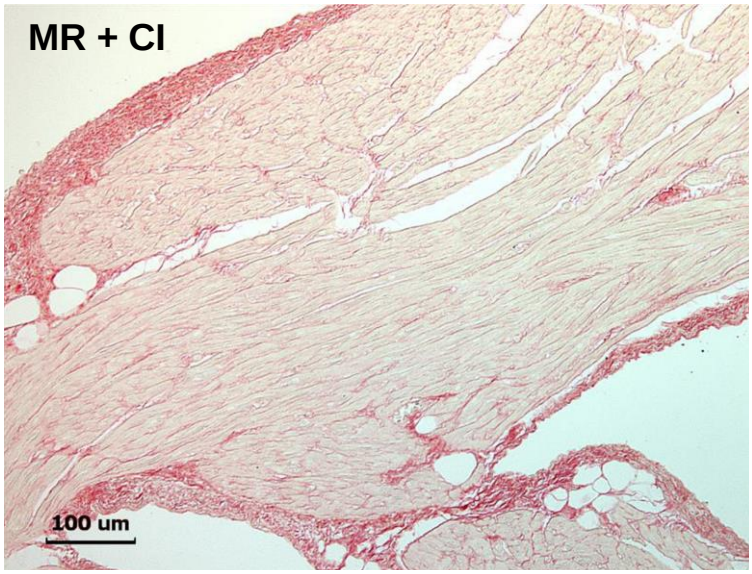
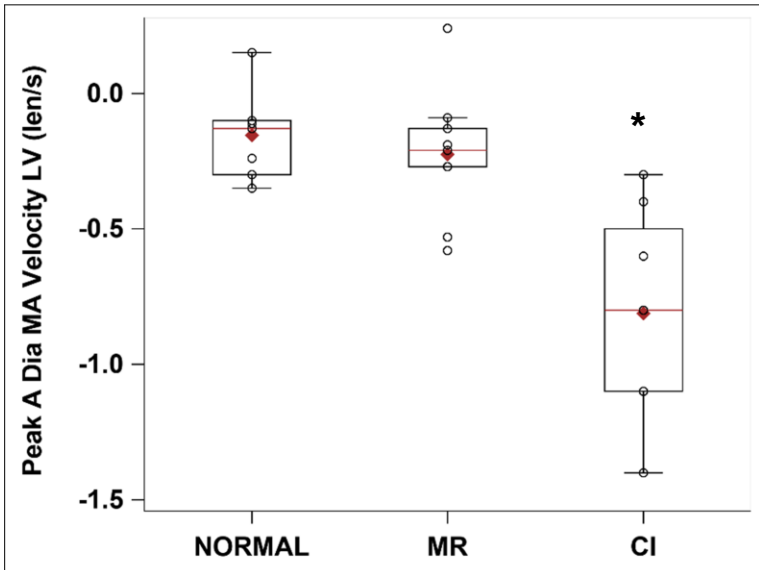


Figure 1. (A) Box plots demonstrating RA and RV (blue) maximum and minimum volume and RA Emptying fraction in MR (n=20) vs. normal volunteers (n=11). (B) RA (red) and RV (blue) time volume curves for the 20 MR patients and 11 normal controls.









Supplemental Figure 2. Picric acid Sirius red staining demonstrates extensive fibro-fatty infiltration in the LA of the MR dog compared to Normal. Chymase inhibition (MR + CI) markedly reduces this structural damage restoring a linear and cross-sectional array of myocytes resulting in improved peak A Mitral Annular (MA) diastolic velocity (bar graph, lower left). * = $p < 0.01$ CI vs Normal and MR.