SUPPLEMENTAL MATERIAL

to

Glycosylated chromogranin A in heart failure - implications for processing and cardiomyocyte calcium homeostasis

Anett Hellebø Ottesen MSc[1,2]; Cathrine R. Carlson PhD[2]; William E. Louch PhD[2]; Mai Britt Dahl MSc[1,3]; Ragnhild A. Sandbu MSc[1,2,3]; Rune Forstrøm Johansen[4]; Hilde Jarstadmarken MSc[2]; Magnar Bjørås PhD[4]; Arne Didrik Høiseth MD, PhD[1]; Jon Brynildsen MD[1]; Ivar Sjaastad MD, PhD; Mats Stridsberg MD, PhD[5]; Torbjørn Omland MD, PhD, MPH[1]; Geir Christensen MD, PhD, MHA[2]; Helge Røsjø MD, PhD[1]

[1]Division of Medicine, Akershus University Hospital, Lørenskog, Norway and K.G. Jebsen Cardiac Research Center and Center for Heart Failure Research, University of Oslo, Oslo, Norway; [2]Institute for Experimental Medical Research, Oslo University Hospital and K.G. Jebsen Cardiac Research Center and Center for Heart Failure Research, University of Oslo, Oslo, Norway; [3]Department of Clinical Molecular Biology, Akershus University Hospital, Lørenskog, Norway and Institute for Clinical Medicine, University of Oslo, Oslo, Norway; [4]Department of Microbiology, Oslo University Hospital, Rikshospitalet, Oslo, Norway, and University of Oslo, Oslo, Norway; [5]Department of Medical Sciences, Uppsala University, Uppsala, Sweden

SUPPLEMENTAL METHODS

CLINICAL STUDY

The clinical studies were approved by the Regional Ethics Committees, performed according to the Declaration of Helsinki, and all participants provided written informed consent before study commencement.

Akershus Cardiac Examination (ACE) 2 Study

In total, 314 patients hospitalized for acute dyspnea were included in the ACE 2 Study at Akershus University Hospital, Lørenskog, Norway. Details of this study have recently been published.[1] In short, patients were included Monday-Thursday 8.00 a.m.-2 p.m., June 2009 - November 2010. Only patients available for blood sampling <24 h following admission were included in the study. Blood sampling was performed by venous puncture with blood samples immediately put on ice, processed in a uniform way, and stored at -80°C until further analyses.

The adjudication committee had access to all clinical data, including follow-up data, but had no knowledge of study biomarker levels, including chromogranin A (CgA) levels. The final diagnosis of acute HF was based on symptoms and clinical signs of HF and evidence of structural or functional myocardial pathology as recommended.[2] The adjudication committee agreed on the final diagnosis in 95% of the patients. Patients were classified by the New York Heart Association (NYHA) functional class, and left ventricular ejection function (LVEF) was recorded from echocardiography reports. Patients with LVEF>50% had evidence of relevant structural heart disease or injury and diastolic dysfunction as also previously reported.[1] Chest radiograph and ECG were recorded. Clinical characteristics, comorbidities, medication, and results on pulmonary testing and blood gas analyses were collected directly from the patients and/ or from the medical records. Patients with non-cardiac condition with life expectancy <1 year and patients unable to provide informed consent were not included in the study. Survival status was obtained from electronic hospital records, which are synchronized with Statistics Norway on a monthly basis with follow-up ending November 1st, 2012.

Biomarker Measurements in Patients

CgA levels were measured in plasma by a commercial enzyme-linked immunosorbent assay (NEOLISA™ Chromogranin A, EuroDiagnostica, Malmö, Sweden). The reference limit of the assay, as provided by the company, is ≤3.0 nmol/L. N-terminal pro-BNP (NT-proBNP) and high-sensitivity troponin T (hs-TnT) levels were measured in plasma by the proBNP II assay (Roche Diagnostics, Penzberg, Germany), and creatinine clearance was estimated by the Cockcroft-Gault formula. Serum levels of cardiac troponin T were measured by a high-sensitivity assay (Elecsys Troponin T high sensitive, Roche Diagnostics).

EXPERIMENTAL STUDIES

All animal experiments were approved by the Norwegian Animal Research Committee, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Post-infarction Heart Failure Mouse Model

Experimental myocardial infarctions were induced by permanent ligation of a main epicardial coronary artery, and the presence of HF was verified by echocardiography 1 week later, as previously reported.[3, 4] After sacrificing HF and sham-operated mice, biopsies from the infarcted and non-infarcted LV, right ventricle, and other organs were collected.

Processing of CgA

The deglycosylation protocol was performed according to the procedure for the Glycoprotein deglycosylation kit (#EDEGLY, Sigma-Aldrich, St. Lois, MO). We used 50 µg total protein lysate.

The samples were treated with different combinations of 5 different deglycosylation enzymes; N-Glycosidase F (5000 U/ml), α2-3,6,8,9-Neuraminidase (5.0 U/ml), Endo-α-N-acetylgalactosaminidase (1.25 U/ml), β1,4-galactosidase (3.0 U/ml), and β-N-Acetylglucosaminidase (45 U/ml). A negative control was included for all deglycosylation samples. Samples were separated through 6 % and 12 % polyacrylamide gels, and proteins were transferred onto a Hybond-P PVDF membrane (Amersham Biosciences Europe, Freiburg, Germany) using a Mini Trans-Blot Cell system (Biorad Laboratories, Hercules, CA, USA). For detection of free peptide CST we separated the samples through a 10-20% Tris Tricine gel (Bio Rad) and subjected the lower part of the membrane to longer exposure to be able to assess immunoblots in the lower range (n=2). The immunostaining was visualized by the use of chemoluminecence; Western Detection System (Amersham Biosciences Europe, Freiburg, Germany) and the ImageReader LAS 3000-mini digital detector system was utilized (Fujifilm, Europe). Antibodies used were anti-CgA (#sc-13090, Santa Cruz Biotechnology, CA, USA) or anti-CST (a gift kindly given from Professor M. Stridsberg, Uppsala University Hospital), and secondary antibody against rabbit (#4030-05, Southern Biotech, Alabama, USA). Relative quantification was performed using ImageJ (NIH). sham: n=15; HF (viable): n=16; HF (infarct): n=6).

Peptide Synthesis

Peptides were synthesized and purified by Genscript (Piscataway, NJ, USA).
Catestatin (CST): SSMKLSFRARAYGFRGPGPQL (human CgA352-372) (>98% purity)
Biotin-CST: RSMRLSFRARGYGFRGPGLQL (bovine CgA344–364) (84% purity). A biotin tag was included at the N-terminus of CTS.
Syntide-2: PLARTLSVAGLPGKK (98.3% purity)
CN21a: KRPPKLGQIGRSKRVVIEDDR (88.9% purity)

Proteins

His-CaMKIIδ (amino acids 69-282, underlined) (Uniprot P15791, 85% purity, custom made from Genscript, Piscataway, NJ):
MHHHHHHENLYFQGKHPNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEADASHCI
QQILESVNHCHLNGIVHRDLKPENLLLASKSKGAAVKLADFGLAIEVQGDQQAWFGFAGTPG
YLSPEVLRKDPYGKPVDMWACGVILYILLVGYPPFWDEDQHRLYQQIKAGAYDFPSPEWDT
VTPEAKDLINKMLTINPAKRITASEALKHPWICQRSTVASMMHRQET

CaMKIIδ (T287D) (full length, underlined) (Uniprot P15791, >70 % purity, custom made from Genscript, Piscataway, NJ):
MHHHHHHENLYFQGMASTTTCTRFTDEYQLFEELGKGAFSVVRRCMKIPTGQEYAAKIINTK
KLSARDHQKLEREARICRLLKHPNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEAD
ASHCIQQILESVNHCHLNGIVHRDLKPENLLLASKSKGAAVKLADFGLAIEVQGDQQAWFGF
AGTPGYLSPEVLRKDPYGKPVDMWACGVILYILLVGYPPFWDEDQHRLYQQIKAGAYDFPSP
EWDTVTPEAKDLINKMLTINPAKRITASEALKHPWICQRSTVASMMHRQETVDCLKKFNARR
KLKGAILTTMLATRNFSAAKSLLKKPDGVKINNKANVVTSPKENIPTPALEPQTTVIHNPDGN
KESTESSNTTIEDEDVKARKQEIIKVTEQLIEAINNGDFEAYTKICDPGLTAFEPEALGNLVEGM
DFHRFYFENALPKINKPIHTIILNPHVHLVGDDAACIAYIRLTQYMDGNGMPKTMQSEETRVW
HRRDGKWQNIHFHRSGSPTVPIKPPCIPNGKENFSGGTSLWQNI

Immunoprecipitation, Pull-down and Surface Plasmon Resonance Experiments

For immunoprecipitation, biotin-CST (30 µM) was incubated with recombinant His-CaMKIIδ (T287D) (5 µg) (Uniprot P15791, >70 % purity, custom made from Genscript, Piscataway, NJ), His antibodies (2.5 µg) (A00186, Genscript), and 100 µl protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology, Dallas, Texas, USA) in 200 µl CaMKII binding buffer (mM: 50 Hepes, pH 7.4, 150 NaCl, 15 $MgCl_2$, 0.1% Triton X-100, 1% BSA, and protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics, Mannheim, Germany) in the presence of $CaCl_2$ (2.5 mM) overnight at 4°C. Negative controls were run without the addition of His-CaMKIIδ (T287D). The beads were then

washed 3 times with 1 ml CaMKII binding buffer. The precipitates were boiled in SDS loading buffer and analyzed by immunoblotting (n=4).

For pull-down experiments, biotin-CST peptide (74 µM), was coupled onto 25 µl anti-biotin agarose beads (A 1559, Sigma Aldrich, St. Louis, MO, USA) in 100 µl CaMKII binding buffer (described above) in the presence of $CaCl_2$ (2.5 mM) at 4°C for 4 hours. Samples were washed once in CaMKII binding buffer, before the addition of recombinant His- CaMKIIδ (T287D) (0.6 µg) (custom made from Genscript, Piscataway, NJ). Negative controls were run without the addition of biotin-CST peptide. Samples were incubated overnight at 4°C, washed three times in CaMKII binding buffer. The precipitates were boiled in SDS loading buffer and analyzed by immunoblotting (n=4).

Antibodies used for immunoblotting were goat anti-CaMKIIδ (A-17) (sc-5392, Santa Cruz Biotechnology, Dallas, Texas, USA) and anti-biotin-HRP (A1085, Sigma Aldrich). Anti-goat IgG (HAF109, R&D, Minneapolis, USA) was used as secondary antibody.

The interaction between CST and CaMKIIδ was also measured by Surface Plasmon Resonance (Biacore 3000, Biacore Inc, Uppsala, Sweden) (n=5). CaMKIIδ was immobilized on CM5 sensor chips (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) until a level of 400 - 4000 resonance units (RU) in flow channel 2 or 4 leaving flow channel 1 or 3 unmodified to provide as reference channels. Five consecutive (single-cycle mode) injections of running buffer (10 mM HEPES pH 7.4 , 150 mM NaCl, 2 mM $CaCl_2$, 1% Triton X-100) at 25ºC and a flow rate of 50 µL/min provided blanks for double-referencing the data. Five concentrations (0.33, 1.00, 3.00, 9.00 and 27.00 µM) of biotin-CST diluted in running buffer were injected in a single-cycle mode at 25ºC with an association phase of 180 s followed by a 180 s flow of running buffer for the dissociation phase. A flow rate of 50 µL/min was used to minimize mass transport effects. Data were analyzed with BIAevaluation software (BIAcore, Piscataway, NJ, USA), and curve fitting was performed with the assumption of one-to-one binding (Langmuir).

CaMKIIδ Activity Assay

CaMKIIδ activity assays were performed using syntide-2 as substrate.[5] Syntide-2 (20 µM) was incubated for 30 min at 30ºC with 133 nM His-CaMKIIδ (T287D) in 10 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM $CaCl_2$, 10 µg/ml calmodulin (CaM; 14-368, Millipore), 5 mM DTT, 0.1 mM ATP, 0.1 µCi/µl [γ-^{33}P]ATP (Hartmann Analytic GmbH, Germany) with or without the presence of 5-40 µM CST. Syntide was omitted in the blank. 5µM or 20 µM of CN21a (a common CaMKII inhibitor)[6] was used as control (n=6-10). The reactions were terminated with 250 mM EDTA on ice and thereafter spotted onto Whatman P81 phosphocellulose chromatography paper (3698-915, Thermo Fisher, Waltham, MA, USA).[7, 8] Free radioactivity was removed by washing the membranes in 1% phosphoric acid under agitation, rinsed in 100% methanol and air dried. Radioactivity was measured by BAS-1800 phosphor imager (Fuji Medical Systems, Stamford, CT, USA).

CaMKIIδ, RyR and Phospholamban Phosphorylation

For preparation of LV samples, mouse hearts were mounted on a Langendorff apparatus as reported above. Hearts were perfused with (i) HT solution and (S)-(-)-Bay K 8644 (BayK; #1546, Tocris Bioscience, Minneapolis, MN) (10 µM) for 20 min, (ii) HT + CST (10 µg/mL) for 10 min followed by HT + CST + BayK for an additional 10 min, (iii) HT + CST (10 µg/mL) for 20 min or (iv) HT alone 20 min. The LV was dissected out, rapidly frozen in liquid nitrogen, and stored at −70°C. Protein was extracted from frozen LV tissue using lysis buffer (mM: 157.5 Sucrose, 1.5 EGTA, 30 NaCl, 22.5 Hepes, 5 EDTA, pH 7.4) with complete protease inhibitor (Roche Diagnostics) and 1% SDS. Protein concentration was measured using a Micro BCA protein assay kit (23235, Thermo Fisher), and samples were separated through 15% or 4-15% Tris-HCl Criterion Precast gels (Bio-Rad Laboratories, Inc., Hercules, CA) and transferred to PVDF membranes (VWR, Radnor, PA, USA). Blots were developed using ECL Plus (RPN2132, GE HealthCare, Buckinghamshire, UK) and visualized using the ImageQuant LAS4000 mini (Fujifilm, Tokyo, Japan). Commercial primary antibodies were used

for pSer2814-RyR2 (A010-31, Badrilla Ltd., Leeds, UK), pSer2808-RyR2 (A010-30, Badrilla), RyR (MA3-925, Thermo Fisher), pSer16-PLB (A010-12, Badrilla), pThr17-PLB (A010-12, Badrilla), PLB (MA3-922, Thermo Fisher), pThr286-CaMKIIδ (ab32678, Abcam), and vinculin (V9131, Sigma Aldrich). Relative quantification was performed using ImageJ (NIH). (Ctr: n=4; CST: n=4; BayK: n=5; CST+BayK: n=4).

Cardiomyocyte Isolation

C57BJ/6 female mice (10-12 weeks old) were anaesthetized (1.8–2.2% isoflurane, 98% oxygen) and sacrificed by cervical dislocation. Excised hearts were cannulated via the aorta, and retrogradely perfused with isolation buffer (mM: 130 NaCl, 25 Hepes, 22 D-glucose, 5.4 KCl, 0.5 $MgCl_2$, 0.4 NaH_2PO_4, pH 7.4) with 0.05 mg/ml collagenase (Worthington, Lakewood, NJ, USA), and 1 mM $CaCl_2$ via a Langendorff setup.[9] After 10-12 min of enzyme perfusion, the LV was minced, and transferred to isolation buffer containing 1% BSA and 0.3 mg deoxyribonuclease. Cardiomyocytes were collected by filtration and sedimentation, the pellet was washed in isolation buffer containing 1% BSA and increasing [Ca^{2+}] (0.1, 0.2 and 0.5 mM).

Ca^{2+}-dependent Fluorescence and Cardiomyocyte Contractions

Measurements of $[Ca^{2+}]_i$ were performed in cardiomyocytes pre-incubated for 10 min with fluo-4 AM (20 mmol/L, Molecular Probes, Eugene, OR), with or without CST (45 nmol/L or 4.5 μmol/L), and then plated on laminin-coated coverslips for additional 10 min. Cells were perfused with Hepes Tyrode's (HT) solution (mM: 140 NaCl, 0.5 $MgCl_2$, 5.0 Hepes, 5.5 glucose, 0.4 NaH_2PO_4, and 5.4 KCl in pH 7.4 at 37°C) containing 5 mM $CaCl_2$ for Ca^{2+} sparks measurements and 1 mM $CaCl_2$ for measurements of transients and contractions.

Ca^{2+} sparks were recorded using an LIVE7 confocal microscope in line-scan mode (Zeiss GmbH, Jena, Germany) with a ×40 water-immersion objective.[10] Ca^{2+} sparks were detected using SparkMaster software.[11] Ca^{2+} transients and contractions were recorded in cells during 0.5, 1, 2 and 5 Hz field stimulation using an Axio Observer / PTI (Zeiss GmbH) with a ×40 oil-immersion objective. Sarcoplasmic reticulum (SR) Ca^{2+} content was assessed by applying caffeine (10 mM, Sigma-Aldrich) and measuring the magnitude of the resulting Ca^{2+} transient. Estimates of the rate constant of decay of the caffeine-induced Ca^{2+} transient served as an estimate of sarcolemmal Ca^{2+} extrusion and the rate constant for SR Ca^{2+} reuptake was calculated as the difference between the steady-state Ca^{2+} transient and the caffeine-induced Ca^{2+} transient (lambda at 1Hz - lambda in caffeine), measured in the same cell over the same magnitude range. Ca^{2+} transients were analyzed with Image J (NIH). Contractions were elicited by field stimulation at 0.5, 1, 2 and 5 Hz with a 3-ms biphasic pulse 50% above threshold and contractions measured using a video-edge detector (Crescent Electronics, Sandy, UT) and Clampex software (Axon Instruments, Union City, CA, USA).
[Ca^{2+} sparks: Ctr: n_{hearts}=3, n_{cells}=12; CST;4.5 μM: n_{hearts}=3, n_{cells}=9, Ca^{2+} sparks: Ctr: n_{hearts}=9, n_{cells}=49; CST; 45 nM: $n_{hearts=}$8, n_{cells}=25; Contractions: Ctr: n_{hearts}=3, n_{cells}=5-12; CST: n_{hearts}=2, n_{cells}=5-8; Ca^{2+} transients: Ctr: n_{hearts}=3, n_{cells}=13; CST: n_{hearts}=4, n_{cells}=7; SR content, 10 mM caffeine: Ctr: n_{hearts}=5, n_{cells}=17; CST: n_{hearts}=4, n_{cells}=15].

Statistics

Data are presented as mean ± SEM apart from biomarker levels that are presented as median (Q 1-3) due to a non-normal distribution, as examined by the Kolmogorov-Smirnov test. Biomarkers were transformed by the natural logarithm (ln) before Cox regression analyses. NYHA functional class was included in the analysis as class IV vs. class II/III. Statistical analyses were performed with SPSS for Windows version 20.0 (SPSS, Chicago, IL) and MedCalc Statistical Software version 14.10.2 (MedCalc for Windows, Mariakerke, Belgium).

Supplemental Table 1. Descriptive statistics on admission for patients hospitalized with dyspnea

	Acute HF (n=143)	Non-HF related dyspnea (n=171)	*P*
Age (y)	75±11	66±15	<0.001
Male sex	90 (63%)	74 (43%)	0.001
Body mass index (kg/m^2)	27±6	26±8	0.79
NYHA† functional class IV	65 (46%)	71 (42%)	0.48
Heart rate (b.p.m.)	92±26	94±20	0.44
Systolic blood pressure (mmHg)	147±32	145±26	0.67
Diastolic blood pressure (mmHg)	82±18	78±15	0.039
Creatinine clearance (mL/min)	66±39	89±50	<0.001
LVEF‡ (%)[a]	41±13	56±7	<0.001
HF*, systolic dysfunction[b]	91 (64%)		
HF*, preserved ejection function	51 (36%)		
History of			
HF*	87 (61%)	14 (8%)	<0.001
Coronary artery disease	78 (55%)	33 (19%)	<0.001
Myocardial infarction	65 (46%)	30 (18%)	<0.001
PCI§	33 (23%)	18 (11%)	0.003
CABG‖	35 (25%)	6 (4%)	<0.001
Hypertension	69 (49%)	51 (30%)	0.001
Atrial fibrillation	68 (48%)	28 (16%)	<0.001
Diabetes mellitus	43 (30%)	25 (15%)	0.001
COPD#	61 (43%)	94 (55%)	0.03
Medication, on admission			
β-blocker	89 (62%)	50 (29%)	<0.001
ACEI**	62 (43%)	25 (15%)	<0.001
ARB††	32 (22%)	27 (16%)	0.14
ACEI or ARB††**	87 (61%)	52 (30%)	<0.001
Aldosterone antagonist	21 (15%)	7 (4%)	0.001
Loop diuretics	97 (68%)	47 (28%)	<0.001
Statin	80 (56%)	49 (29%)	<0.001
Warfarin	53 (37%)	22 (13%)	<0.001
ASA‡‡	70 (49%)	52 (30%)	0.001
Clopidogrel	17 (12%)	6 (4%)	0.005
Digitalis	20 (14%)	5 (3%)	<0.001
Slow release nitrate	18 (13%)	11 (6%)	0.06
Proton pump inhibitor	32 (22%)	30 (18%)	0.28
Short-acting β_2-agonist, inhalation	41 (29%)	66 (39%)	0.07
Ipratropium bromide	32 (22%)	62 (36%)	0.07
Long-acting β_2-agonist, inhalation	42 (29%)	67 (39%)	0.07

Tiotropium bromide	10 (7%)	23 (14%)	0.06
Corticosteroids, inhalation	36 (25%)	61 (36%)	0.045
Corticosteroids, oral	14 (10%)	32 (19%)	0.026
Insulin	19 (13%)	13 (8%)	0.10
Anti-diabetic medication, oral	25 (18%)	15 (9%)	0.02
CgA, admission (nmol/L)	2.6 (1.5-5.5)	1.9 (1.2-3.0)	<0.001
CST, admission (nmol/L)	1.2 (1.1-1.4)	1.2 (1.0-1.4)	0.34
CgA:CST (%)[c]	57 (23-78)	41 (0-65)	0.001
hs-TnT[§§]**, admission (ng/L)**	38 (22-75)	13 (4-25)	<0.001
NT-proBNP[‖‖]**, admission (pg/mL)**	3588 (1578-8404)	348 (119-1139)	<0.001

Categorical variables are presented as counts (percentage) and continuous variables as mean±SD except biomarker concentrations that are reported as median (quartile 1-3) due to a right-skewed distribution

[*]HF, heart failure; [†]NYHA, New York Heart Association; [‡]LVEF, left ventricular ejection function; [§]PCI, percutaneous coronary intervention; [‖]CABG, coronary artery by-pass grafting; [#]COPD, chronic obstructive pulmonary disease; [**]ACEI, ACE-inhibitor; [††]ARB, Angiotensin II receptor blocker; [‡‡]ASA, acetyl salicylic acid; [§§]hs-TnT, high-sensitivity troponin T; [‖‖]NT-proBNP, N-terminal pro-B-type natriuretic peptide

[a]Available in the acute HF patients (n=143) and a subgroup of non-HF patients (n=105)

[b]LVEF<50%

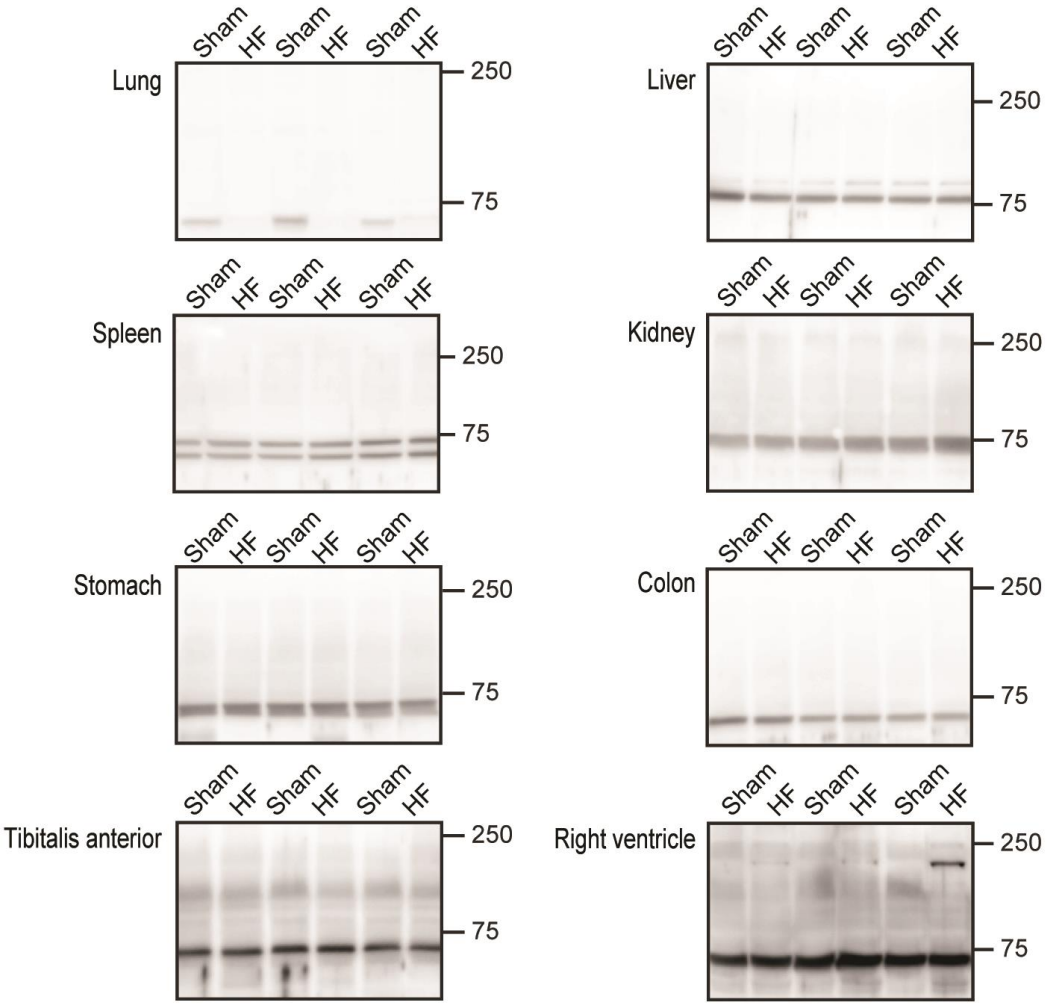
[c]Calculated by this formula: (CgA-CST/CgA) x 100

Supplemental Table 2. Correlations between CgA and NT-proBNP and clinical variables in the acute HF patients (n=143)

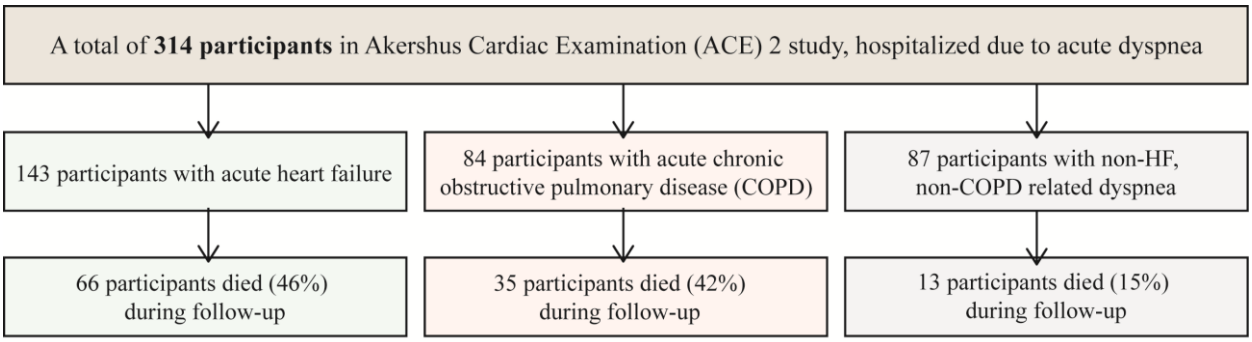
	CgA		CST		NT-proBNP	
	r	**p**	**r**	**p**	**r**	**p**
Age	0.20	0.02	-0.21	0.014	0.21	0.01
[*]**BMI**	0.02	0.83	-0.003	0.97	-0.30	<0.001
Creatinine clearance	-0.50	<0.001	0.12	0.16	-0.47	<0.001
Heart rate, admission	-0.03	0.72	-0.16	0.05	0.05	0.53
Systolic blood pressure	-0.16	0.05	-0.006	0.95	-0.04	0.61
Diastolic blood pressure	-0.30	<0.001	-0.002	0.98	0.005	0.95
[†]**LVEF**	-0.003	0.98	-0.035	0.68	-0.41	<0.001
CgA	-		-0.047	0.58	0.29	<0.001
CST	-0.047	0.58	-		-0.14	0.11
hs-TnT	0.27	0.001	-0.12	0.14	0.43	<0.001
NT-proBNP	0.29	<0.001	-0.14	0.11	-	
[‡]**NE**	0.03	0.74	0.15	0.07	0.23	0.007

[*]BMI, body mass index; [†]LVEF, left ventricular ejection function; [‡]NE, norepinephrine

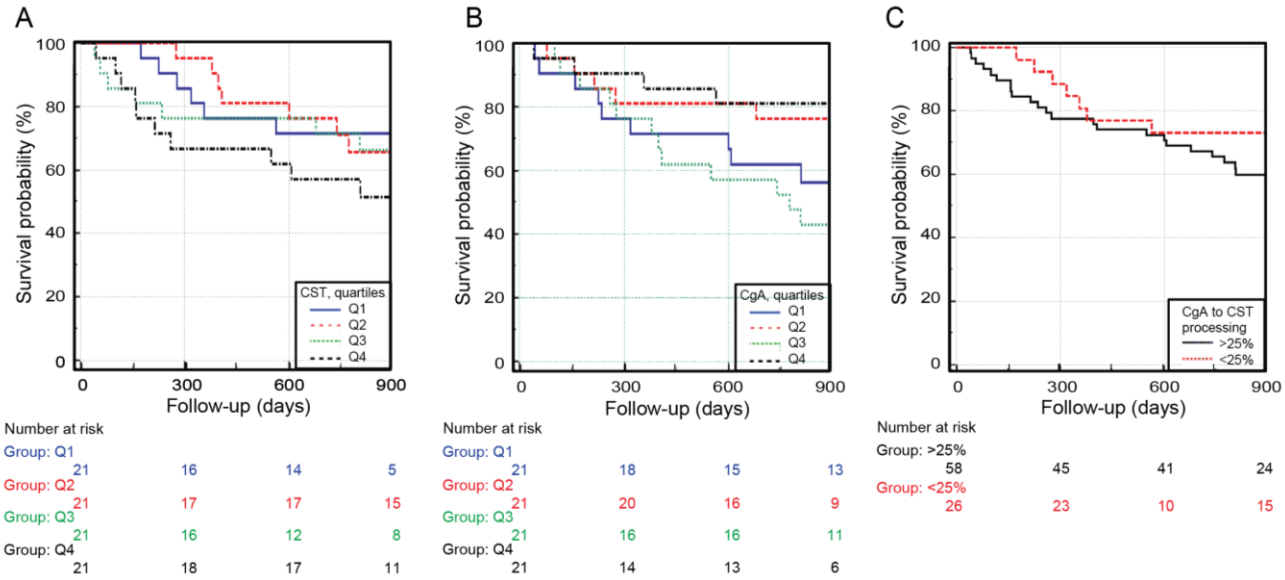
Supplemental Figure 1. High molecular weight (hmw) CgA bands were found in the left and right ventricle of HF animals, but not in sham-operated animals or in organs outside of the heart



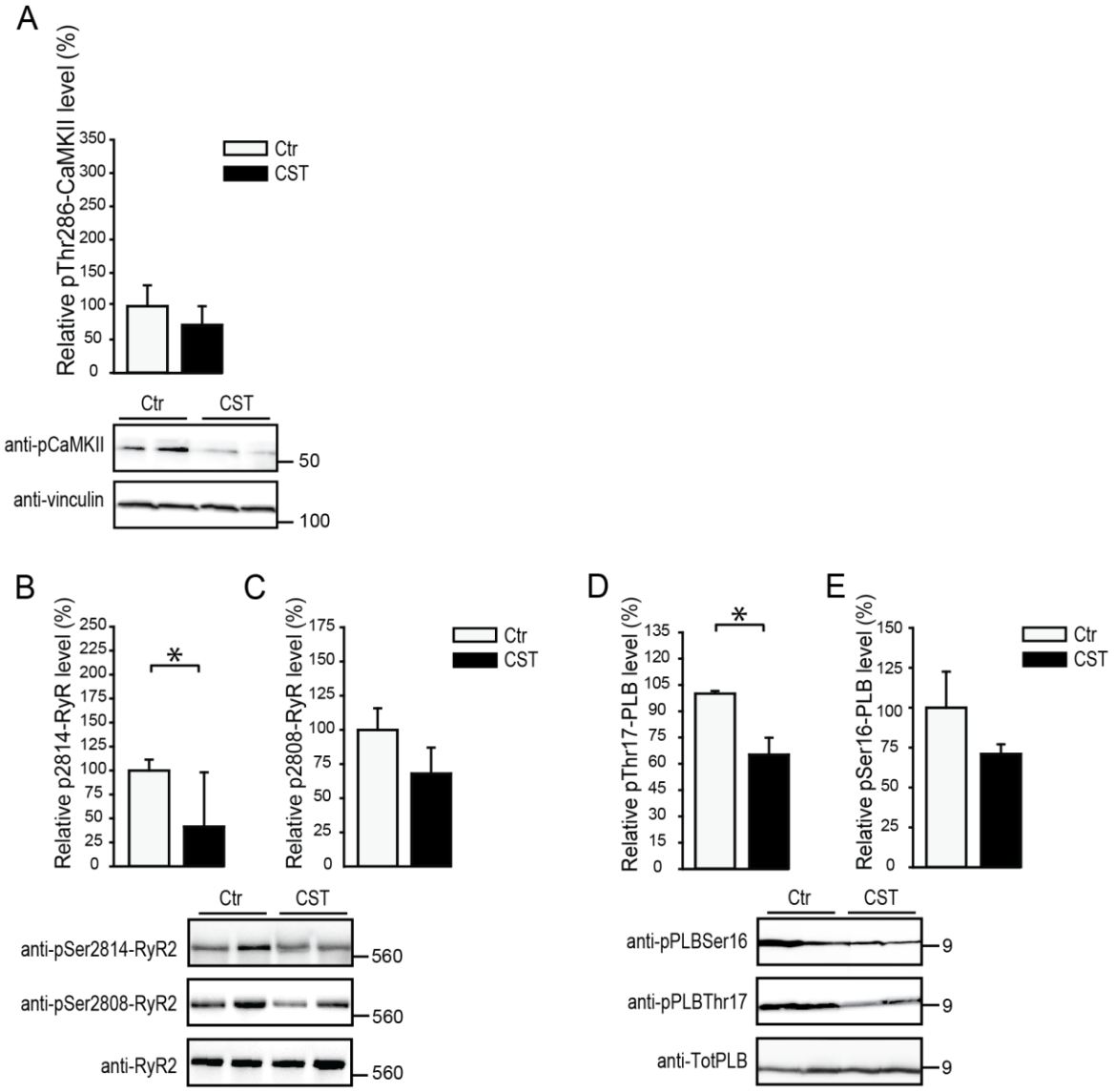
Supplemental Figure 2. Flow chart of patient inclusion in Akershus Cardiac Examination (ACE) 2 study.



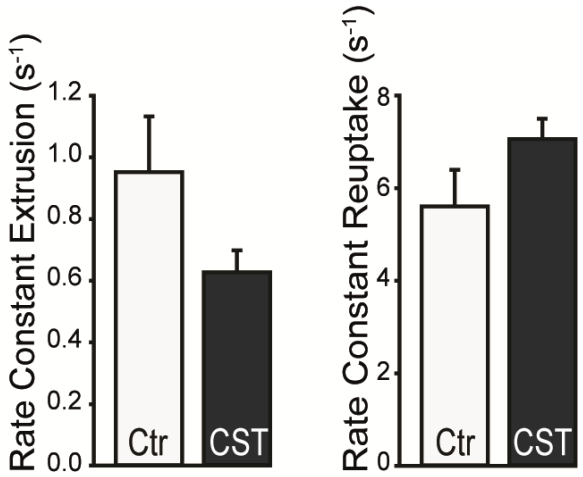
Supplemental Figure 3. (A) CgA levels, **(B)** CST levels, and **(C)** CgA to CST conversion were not associated with mortality in the patients hospitalized with acute exacerbation of COPD.



Supplemental Figure 4. (A) Autophosphorylation of Thr286-CaMKII in CST-perfused hearts was not altered. **(B)** CST reduced basal Ser2814-RyR2 phosphorylation, **(C)** but not Ser2808-RyR2 phosphorylation. **(D)** CST reduced basal CaMKII-dependent phosphorylation of Thr17-PLB, and **(E)** BayK-induced Ser16-PLB phosphorylation. (Ctr: n=4; CST: n=4) *$p \leq 0.05$.



Supplemental Figure 5. CST treatment did not alter rates of Ca^{2+} extrusion from the cell or Ca^{2+} reuptake into SR.



SUPPLEMENTAL REFERENCES

1. Røsjø H, Dahl MB, Jørgensen M, Røysland R, Brynildsen J, Cataliotti A, Christensen G, Høiseth AD, Hagve TA and Omland T. Influence of Glycosylation on Diagnostic and Prognostic Accuracy of N-Terminal Pro-B-Type Natriuretic Peptide in Acute Dyspnea: Data from the Akershus Cardiac Examination 2 Study. *Clinical Chemistry*. 2015;61:1087-97.
2. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, Falk V, Filippatos G, Fonseca C, Gomez-Sanchez MA, Jaarsma T, Kober L, Lip GY, Maggioni AP, Parkhomenko A, Pieske BM, Popescu BA, Ronnevik PK, Rutten FH, Schwitter J, Seferovic P, Stepinska J, Trindade PT, Voors AA, Zannad F, Zeiher A and Guidelines ESCCfP. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur Heart J*. 2012;33:1787-847.
3. Røsjø H, Husberg C, Dahl MB, Stridsberg M, Sjaastad I, Finsen AV, Carlson CR, Øie E, Omland T and Christensen G. Chromogranin B in heart failure: a putative cardiac biomarker expressed in the failing myocardium. *Circulation Heart Failure*. 2010;3:503-11.
4. Røsjø H, Stridsberg M, Florholmen G, Stensløkken KO, Ottesen AH, Sjaastad I, Husberg C, Dahl MB, Øie E, Louch WE, Omland T and Christensen G. Secretogranin II; a protein increased in the myocardium and circulation in heart failure with cardioprotective properties. *PloS ONE*. 2012;7:e37401.
5. Chang BH, Mukherji S and Soderling TR. Characterization of a calmodulin kinase II inhibitor protein in brain. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95:10890-5.
6. Vest RS, Davies KD, O'Leary H, Port JD and Bayer KU. Dual mechanism of a natural CaMKII inhibitor. *Mol Biol Cell*. 2007;18:5024-33.
7. Witt JJ and Roskoski R, Jr. Rapid protein kinase assay using phosphocellulose-paper absorption. *Anal Biochem*. 1975;66:253-8.
8. Asensio CJ and Garcia RC. Determination of a large number of kinase activities using peptide substrates, P81 phosphocellulose paper arrays and phosphor imaging. *Anal Biochem*. 2003;319:21-33.
9. Louch WE, Sheehan KA and Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. *Journal of Molecular and Cellular Cardiology*. 2011;51:288-98.
10. Louch WE, MØrk HK, Sexton J, Strømme TA, Laake P, Sjaastad I and Sejersted OM. T-tubule disorganization and reduced synchrony of Ca2+ release in murine cardiomyocytes following myocardial infarction. *J Physiol*. 2006;574:519-33.
11. Picht E, Zima AV, Blatter LA and Bers DM. SparkMaster: automated calcium spark analysis with ImageJ. *American journal of physiology Cell physiology*. 2007;293:C1073-81.