Published in final edited form as:

Int J Clin Exp Med. 2017 ; 10(1): 1051–1058.

Upregulation of autophagy genes and the unfolded protein response in human heart failure

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Abstract

The cellular environment of the mammalian heart constantly is challenged with environmental and intrinsic pathological insults, which affect the proper folding of proteins in heart failure. The effects of damaged or misfolded proteins on the cell can be profound and result in a process termed “proteotoxicity”. While proteotoxicity is best known for its role in mediating the pathogenesis of neurodegenerative diseases such as Alzheimer’s disease, its role in human heart failure also has been recognized. The UPR involves three branches, including PERK, ATF6, and IRE1. In the presence of a misfolded protein, the GRP78 molecular chaperone that normally interacts with the receptors PERK, ATF6, and IRE-1 in the endoplasmic reticulum detaches to attempt to stabilize the protein. Mouse models of cardiac hypertrophy, ischemia, and heart failure demonstrate increases in activity of all three branches after removing GRP78 from these internal receptors. Recent studies have linked elevated PERK and CHOP in vitro with regulation of ion channels linked with human systolic heart failure. With this in mind, we specifically investigated ventricular myocardium from 10 patients with a history of conduction system defects or arrhythmias for expression of UPR and autophagy genes compared to myocardium from non-failing controls. We identified elevated *Chop, Atf3*, and *Grp78* mRNA, along with XBP-1-regulated *Cebpa* mRNA, indicative of activation of the UPR in human heart failure with arrhythmias.

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Disclosure of conflict of interest

None.

Keywords

Heart failure; unfolded protein response; GRP78; IRE-1; CHOP

Introduction

The cellular environment of the mammalian heart constantly is challenged with environmental and intrinsic pathological insults, which affect the proper folding of proteins in human heart failure [1]. The effects of damaged or misfolded proteins on the cell can be profound and result in a process termed “proteotoxicity”. While proteotoxicity is best known for its role in mediating the pathogenesis of neurodegenerative diseases such as Alzheimer’s disease, its role in human heart failure also has been recognized [2]. Of note, both exercise [3] and direct induction of autophagy [4] experimentally attenuate both the accumulation of soluble misfolded pre-amyloid oligomers and the heart failure it induces [5, 6].

The unfolded protein response (UPR) has been studied extensively in diabetes and neurological diseases as a response to the presence and/or accumulation of damaged proteins in the endoplasmic reticulum. The UPR stress response is highly conserved from yeast to mammalian cells and has a protective role in cell survival by eliminating misfolded proteins. However, when its activation is prolonged, the UPR can induce cell dysfunction and death [7]. In the heart, the UPR is not well understood and has been implicated in both protecting and impairing heart function in experimental systems [7].

The UPR involves three branches, each with distinct signaling pathways that enhance protein folding or attenuate protein synthesis and loading of the endoplasmic reticulum (1) PERK; (2) ATF6; and (3) IRE1 [8]. In the presence of a misfolded protein, the GRP78 molecular chaperone that normally interacts with the receptors PERK, ATF6, and IRE-1 in the endoplasmic reticulum detaches to attempt to stabilize the protein [8]. Activation of PERK, ATF6, and IRE-1 occurs upon removal of GRP78. In mouse models of cardiac hypertrophy [9–11], ischemia [12, 13], and heart failure [10, 14], elevations in all three UPR pathways have been reported. In human failing heart tissue, elevated PERK and down-stream CHOP mRNA has been reported from 3 human heart failure samples [15], with elevated spliced variant XBP1 and downstream GRP78 proteins levels reported in 4 human dilated and ischemic cardiomyopathy samples [16]. Interestingly, the identification of elevated PERK and CHOP led to in vitro studies linking PERK activation to SCN5a and Kv4.3 mRNA levels, implicating a link to the sodium current in human systolic heart failure. Systolic heart failure greatly increases susceptibility to arrhythmias, and ventricular arrhythmia is a common cause of death in heart failure patients. With this in mind, we specifically investigated ventricular myocardium from 10 patients with a history of conduction system defects or arrhythmias for expression of UPR and autophagy genes compared to myocardium from non-failing controls.

Methods

Human heart failure samples and institutional review board (IRB) approval

Human samples are from subjects consented and collected for future research by the Duke Human Heart Repository (Pro00005621). A “Request for Waiver or Alteration of Consent and HIPAA Authorization” was submitted to the Duke IRB for the present study (Pro00060625) and approved (19 February 2015). In parallel, a request for “Exemption from IRB Review” was submitted to the University of North Carolina IRB for the present studies to be performed at UNC (14-3334) and approved (10 March 2015). Heart failure samples were de-identified without PHI and collected from heart transplant recipients at the time of transplant. Non- failing control samples were obtained from organ donors whose hearts were not used for transplant. De-identified heart samples were chosen based on the presence of cardiac conduction system abnormality or arrhythmia, which included prolonged QT, right or left bundle branch block, ventricular tachycardia, and other dysrhythmias, as indicated in Table 2.

RNA isolation and qRT-PCR analysis of mRNA

Cardiac tissues were homogenized using a TissueLyser LT (Qiagen N.V. #69980, Venlo, The Netherlands) according to the manufacturer’s protocols. Approximately 20–40 mg of apical ventricle was homogenized in 1 mL of Trizol (Life Technologies #15596-026, Carlsbad, CA) using a 5-mm stainless steel bead (Qiagen N.V. #69989). Chloroform (200 μL) was added, centrifuged at 12,000 g (15 min at 4°C), isopropanol (0.5 mL) was then added to the aqueous phase, centrifuged at 12,000 g (10 min at 4°C), and the resulting RNA pellet was washed with 1 mL of 75% ethanol, then centrifuged at 7500 g (5 min at 4°C). The resulting pellet was dried and resuspended in RNase-free water. RNA concentrations were then determined by UV spectroscopy (absorbance of 260–280 nm). RNAs (500 ng) were reverse-transcribed using iScript reverse transcription supermix (Bio-Rad Laboratories #170-8841, Hercules, CA). TaqMan gene expression assays were performed using universal TaqMan master mix (Life Technologies #4304437) using the fourteen primer pairs shown in Table 1.

Statistical analysis

SigmaPlot (Systat Software, Inc., San Jose, CA) was used to determine significant statistical difference by Student’s t-test. A P value < 0.05 was considered significant.

Results

The unfolded protein response (UPR) includes signaling by IRE-1, PERK, and ATF6 that are activated when misfolded proteins displace GRP78 from these receptors in the endoplasmic reticulum [17, 18]. We investigated how IRE-1 signaling was affected by measuring spliced *Xbp-1* [18] and the XBP-1 regulated expression of the C/EBP (*Cebpa*) gene expression [19]. Compared to non-failing human heart controls, samples from human heart failure patients did not have increased spliced *Xbp-1* mRNA (Figure 1A). However, significant increases in *hCebpa* mRNA were identified (Figure 2B), consistent with increased IRE-1 pathway signaling. The relationship between XBP1 and C/EBPb (*Cebpb*) is a bit more complex, with evidence that C/EBPb induces expression of XBP1 protein [19].

The heart failure samples did not demonstrate any differences in *hCebpb* mRNA from controls (Figure 2B).

The stress caused in the ER by the presence of unfolded proteins can also cause signaling through PERK, whereby PERK proteins dimerize and undergo autophosphorylation [20]. This autophosphorylation leads to downstream phosphorylation of eIF2 and elevations in CHOP expression by qRT-PCR [21]. We identified significant increases in both *Chop* and *Atf3* mRNA in the human heart failure samples (Figure 1C). Activation of ATF4 increases target gene expression, including the transcription factor CHOP (CCAAT/-enhancer-binding protein homologous protein) [22], which itself induces the expression of *Atf3* mRNA [23]. While activation of ATF6 signaling was not directly measured, due largely to the deficiencies in reliable reagents [22], we determined the mRNA levels of other reported genes involved in the UPR. We identified significant increases in *Grp78* (Figure 1D), likely reflecting compensatory upregulation in response to the clear activation of GRP78 and IRE-1 signaling [22]. In summary, the significant increases in *Cebpa, Chop, Atf3*, and *Grp78* mRNA seen in human heart failure provide evidence for activation of the IRE-1 and PERK signaling pathways.

In neurodegenerative diseases (e.g. Alzheimer Disease's) increases in autophagy ameliorate endoplasmic reticulum stress and the UPR by eliminating misfolded proteins [24]. Both abnormal UPR and impaired autophagy have been implicated in the heart experimentally and are important complementary systems for reacting to misfolded proteins [25]. We therefore investigated the transcriptional regulation of autophagy in these failing human hearts. Central to the regulation of autophagy in heart disease, the Beclin 1 protein functions as a scaffolding protein assembling the Beclin interactome to regulate Class II PI3K/VPS34 activity, which tightly controls autophagy at multiple stages [26]. Autophagy is also regulated at the transcriptional level: increased *Atg5, Atg7, Atg12, Bnip3*, and *Vps34* mRNA enhance autophagy [27]. RT-qPCR analysis of these genes in human heart failure identified significantly increased *Beclin1* and *Vps34* mRNA (Figure 2A), along with increased *Atg5* and *Atg7* mRNA (Figure 2B), consistent with increased autophagy.

Discussion

Recent studies have identified the presence of non-functional sodium channels (SCN5A) in heart failure patients, which then get trapped in the endoplasmic reticulum and activate the UPR [15]. Induction of SCN5a variants by angiotensin II or hypoxia activates the UPR to down-regulate the Na+ channel activity, all of which is prevented by inhibiting PERK [15], demonstrating the causal relationship between these SCN5a variants, the UPR, and Na+ channel activity. The activation of the UPR has been reported in dilated (n=21) and ischemic cardiomyopathy patients (n=21) compared to non-failing control hearts, including increases in GRP78, XBP1, and ATF6 [28]. Other studies have identified increases in PERK and CHOP protein levels along with increased mRNA of proteins involved in IRE-1 (increased *Cebpa*) and CHOP (*Chop, Atf3*) in heart failure patients (n=3) [15]. Given the close relationship between arrhythmias and the UPR, we interrogated myocardium from heart failure patients with arrhythmias to determine activation of the UPR in the current study. We identified significant increases in myocardial *Cebpa, Chop, Atf3*, and *Grp78* mRNA in

human heart failure providing evidence for activation of the IRE-1 and PERK signaling pathways. In addition, we identified evidence for increases in autophagy in tissue from these patients. Conclusive demonstration of autophagic flux is essentially impossible in human tissue, but these transcriptional changes confirm that the autophagic signaling pathway is intact and active.

The relationship between endoplasmic reticulum stress, activation of the UPR, and autophagy has been described experimentally to be a form of cardioprotection. In previous studies, drugs routinely given to induce the UPR (tunicamycin and thapsigargin) were given at varying doses prior to ischemia-reperfusion injury using a working heart preparation (30 min ischemia followed by 2 hours reperfusion) [29]. These studies found that lower doses of tunicamycin and thapsigargin led to autophagy and induction of GRP78, and were associated with improved function, reduced infarct size, and decreased apoptosis after ischemia-reperfusion [29]. These studies demonstrated the proof of concept that induction of autophagy by endoplasmic reticulum stress may enhance the cardiac response to injury. In the context of human heart failure, the induction of the UPR and autophagy in this study of samples from end-stage human heart failure patients may represent attempts at cardioprotection after chronic extreme injury.

Cardiac insults, including oxidized proteins, induce the UPR, which includes the endoplasmic reticulum chaperone protein GRP78 [30]. GRP78 is found in the endoplasmic/sarcoplasmic reticulum bound to PERK, IRE1, and ATF6 receptors to prevent their activation of the UPR [21]. In the presence of unfolded proteins, GRP78 preferentially shifts to these proteins to stabilize their conformation, leaving PERK, IRE1, and ATF6 to regulate survival and apoptotic signaling downstream [21]. Previous studies have suggested that endoplasmic reticulum stress may be a therapeutic target in heart disease, as XBP-1 mediated induction of endoplasmic reticulum chaperones occurs in ischemia reperfusion injury and PERK/CHOP transmits pro-apoptotic signaling [31]. However, their regulation in heart failure has been described in humans only experimentally with increased SERCA expression [32].

Acknowledgments

We would like to thank Dr. Hyung-Suk Kim for assistance with primer design of the RT qPCR probes (UNC Animal Clinical Chemistry Core Facility) and Michael Watson of the Human Heart Repository (Duke University) for support obtaining the human tissue used in the present study. This work was supported by funding from the National Institutes of Health (RO1HL104129 to M.W. and CA125237 to S.B.), a Jefferson-Pilot Corporation (fellowship to M.W.), and the Leducq Foundation (to M.W.).

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Abbreviations

CHOP	CCAAT/-enhancer-binding protein homologous protein
IRE-1	inositol-requiring enzyme 1
GRP78	glucose-regulated protein 78 kDa
UPR	unfolded protein response
XBP-1	Xbox binding protein.

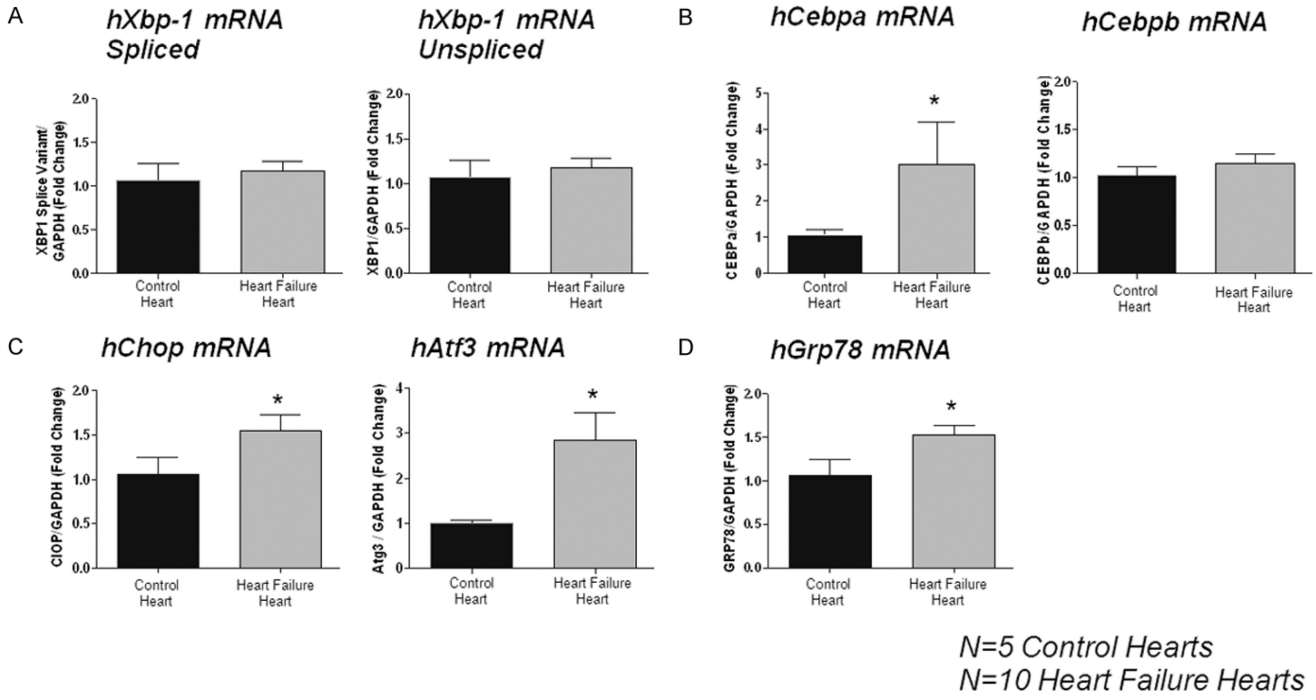

Figure 1.

Transcriptional regulation of the unfolded protein response in human heart failure. qRT-PCR analysis of genes involved in the unfolded protein response normalized to *Gapdh* in ventricular myocardium from patients with conduction defects or arrhythmias (n=10) and control healthy hearts (n=5). Patients' clinical data are detailed in Table 2. Data are presented as means ± SEM with significant differences indicated (*, $P < 0.05$).

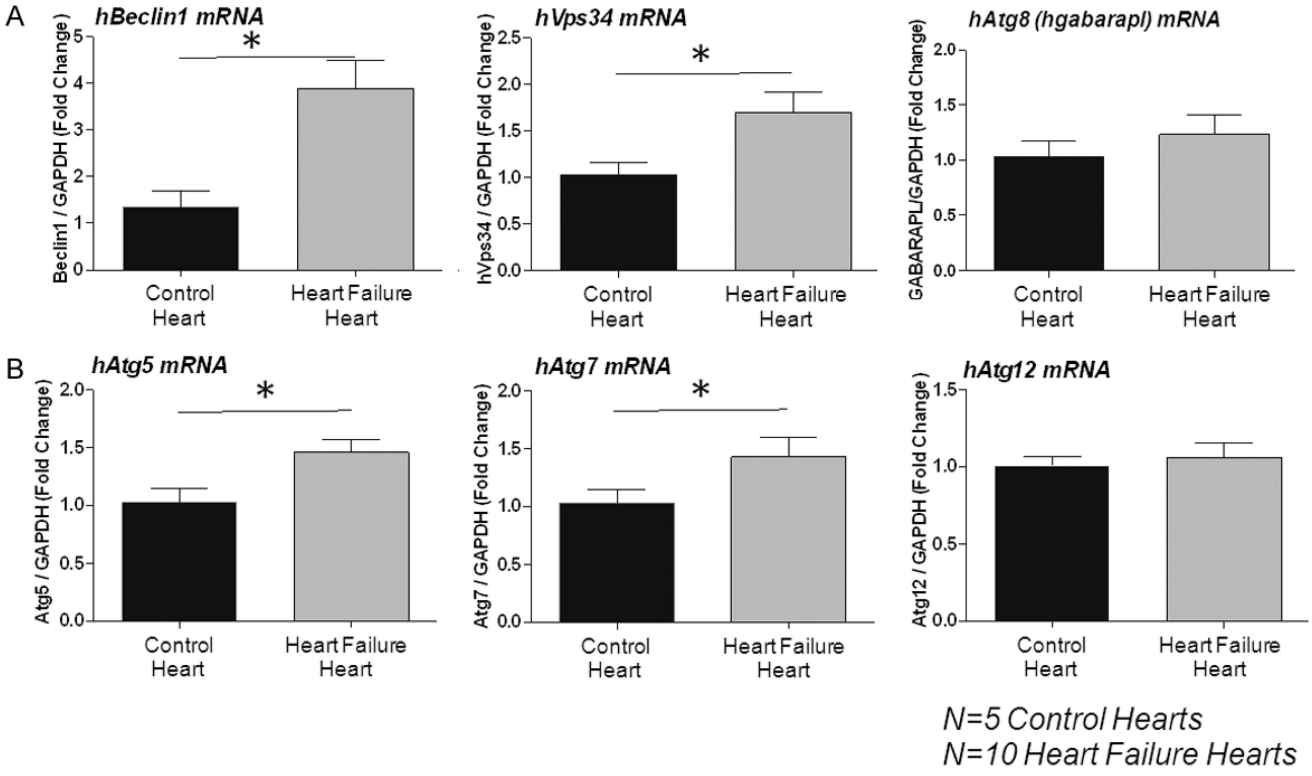

Figure 2.

QRT-PCR analysis of autophagy genes in human heart failure. qRT-PCR analysis of genes involved in autophagy normalized to Gapdh in ventricular myocardium from patients with conduction defects or arrhythmias (n=10) and control healthy hearts (n=5). Patients' clinical data are detailed in Table 2. Data are presented as means ± SEM with significant differences indicated (*, $P < 0.05$).

Table 1

Primer Probe sets used on human heart samples

QRT-PCR Primer and Probe sets (human):
Atg5
Forward: CAA GAA GAC ATT AGT GAG ATA TGG
Reverse: GCA AGA AGA TCA AAT AGC AAA CC
Probe: FAT ATG AAG GCA CAC CAC TGA AAT GGC AQ
Atg7
Forward: AGC AGC AGT GAC GAT CGG AT
Reverse: CGT GAA AGA AAT CCC CGG AT
Probe: FTG AGC CTC CAA CCT CTC TTG GGC Q
Atg12
Forward: TAT GTG AAT CAG TCC TTT GCT C
Reverse: CCA GTT TAC CAT CAC TGC CA
Probe: FTC CCC AGA CCA AGA AGT TGG AAC TCQ
Beclin1
Forward: GCA TAT GGC ATG ATA GCC TC
Reverse: TCC ACA TGG CAT TAA TCT CCT
Probe: FAT CAC CAG TAT CTT CAG CCC CAG GQ
Vps34
Forward: GCA CTT GAA CCA GAT AAA ACT GT
Reverse: GCA TGT AAT GCA CAG CCT CT
Probe: FTC CGA CAG GTC TAA GCG GAA TTT ATC CQ
Gabarapl1
Forward: GAC GCC TTA TTC TTC TTT GTC A
Reverse: CTC ATG ATT GTC CTC ATA CAG
Probe: FAC CAT CCC TCC CAC CAG TGC TAC CAQ
Grp78
Forward: GCT TCT GAT AAT CAA CCA ACT G
Reverse: TGT ACC CAG AAG ATG ATT GTC
Probe: FAG GTC TAT GAA GGT GAA AGA CCC CTG Q
Xbp-1 Spliced
Forward: GAA GAG GAG GCG GAA GCC AA
Reverse: GCC TGC ACC TGA CTC AGC A
Xbp-1 Full
Forward: GAA GAG GAG GCG GAA GCC AA
Reverse: TAG TCT GAG TGC TGC GGA CT
Probe: FAC CCG GCC ACT GGC CTC ACT TCA Q
Cebpa
Forward: AGG CTC GCC ATG CCG GGA
Reverse: GCT CCG CCT CGT AGA AGT
Probe: FAC TCT AAC TCC CCC ATG GAG TCG GQ

QRT-PCR Primer and Probe sets (human):
Cebpb
Forward: ATA AAT AAC CGG GCT CAG GAG
Reverse: CCT CGG GTG GGT CCC CTT
Probe: FTT AGC GAG TCA GAG CCG CGC ACG Q
Chop
Forward: CTG GAA ATG AAG AGG AAG AAT C
Reverse: TGG TTC TGG CTC CTC CTC A
Probe: FTC ACC ACT CTT GAC CCT GCT TCT CTG Q
Atf3
Forward: TGC CTG CAG AAA GAG TCG GA
Reverse: CTT CTC GTT CTT GAG CTC CT
Probe: FCT GAG CCT TCA GTT CAG CAT TCA CAC Q
Gapdh
Forward: ACC TCA ACT ACA TGG TTT AC
Reverse: GAA GAT GGT GAT GGG ATT TC
Probe: FCA AGC TTC CCG TTC TCA GCC Q

F: 5'-Fluorescein (FAM); Q: Quencher (TAMRA). The method for RNA isolation and Real-time RT-PCR was described in *Kim et al, 2002 PNAS 99: 4602–4607*.

Table 2

Patient clinical characteristics with arrhythmia description

Group	Age	Sex	Clinical Characteristics	Arrhythmia
NonF 1	66	F	Cerebral aneurysm; nonsmoker; not diabetic; heart normal size; BMI=24	None
NonF 2	54	F	EF=55%; no hypertrophy; nonsmoker; nondiabetic; BMI=30	None
NonF 3	25	M	HTN; ICH/stroke	None
NonF 4	62	M	HTN; ICH/stroke	None
NonF 5	36	M	EF=55%	None
HF 1	59	M	s/p CABG; pulmonary edema; NIDDM; HTN; CAD	Prolonged QT
HF 2	62	F	ICM; CAD; HTN; s/p ICD; s/p IABP; smoker; anterior STEMI w/ occlusion of proximal LAD; COPD	Bradycardia
HF 3	58	M	EF 10%; DCM; COPD; CAD; PE; s/p ICD; NIDDM; previous MI; s/p RVAD; ex-smoker; cardiac arrest	LBBB; VF
HF 4	75	F	ICM; Acute MI with cardiogenic shock (NSTEMI); NYHA Class IV; s/p ECMO; IDDM; acute renal failure	RBBB
HF 5	61	M	EF 15%; NICM; diabetes DM2 (NIDDM); HTN; CAD; Cardiogenic shock; CKD; former smoker (quit 1994); COPD; NYHA Class IV; s/p ICD; atrial fibrillation	AF; NSVT
HF 6	57	M	EF 15-20%; NICM; s/p IABP; traumatic brain injury; HTN; hypercalcemia; obese; OSA; hyponatremia; cardiogenic shock; s/p ICD; pulmonary HTN; former smoker	AF; Dysrhythmias
HF 7	64	M	NICM; s/p LVAD; HTN; Mitral valve disorder; ICD; former smoker; non-diabetic; BMI=26	Paroxysmal VT
HF 8	61	M	EF < 15%; ICM; NSTEMI; s/p IABP; s/p ICD; CAD; PVD; BMI=29; former smoker	Polymorphic VT
HF 9	49	F	DCM; s/p LVAD; cardiogenic shock; HTN; BMI=20; inotrope support; AR, MR, Pulmonary HTN; former smoker	Dysrhythmia
HF 10	46	M	NICM; acute kidney injury; cardiogenic shock; s/p ICD; pulmonary HTN; s/p IABP and inotropes; acute myocarditis	LBBB

AF=atrial fibrillation; AR=aortic regurgitation; BMI=body mass index; CABG=coronary artery bypass grafting; CAD=coronary artery disease; CKD=chronic kidney disease; COPD=chronic obstructive pulmonary disease; DCM=dilated cardiomyopathy; ECMO=extracorporeal membrane oxygenation; EF=ejection fraction; HF=Heart Failure; HTN=hypertension; IABP=intra-aortic balloon pump; ICD=implantable cardioverter defibrillator; ICH=intracerebral hemorrhage; ICM=ischemic cardiomyopathy; LAD=left anterior descending coronary artery; LBBB=left bundle branch block; MR=mitral regurgitation; NIDDM=non-insulin dependent diabetes mellitus; (N) STEMI=(non) ST elevation myocardial infarction; s/p=status post; NSVT=non-sustained ventricular tachycardia; NYHA=New York Heart Association; NICM=non-ischemic cardiomyopathy; NonF=Non-failing control; OSA=obstructive sleep apnea; PE=pulmonary embolism; PVD=peripheral vascular disease; RBBB=right bundle branch block; R/LVAD=right/left ventricular assist device; VF=ventricular fibrillation; VT=ventricular tachycardia.