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Secretoneurin Is a Novel Prognostic Cardiovascular Biomarker Associated With Cardiomyocyte Calcium Handling

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ABSTRACT

BACKGROUND Secretoneurin (SN) levels are increased in patients with heart failure (HF), but whether SN provides prognostic information and influences cardiomyocyte function is unknown.

OBJECTIVES This study sought to evaluate the merit of SN as a cardiovascular biomarker and assess effects of SN on cardiomyocyte Ca^{2+} handling.

METHODS We assessed the association between circulating SN levels and mortality in 2 patient cohorts and the functional properties of SN in experimental models.

RESULTS In 143 patients hospitalized for acute HF, SN levels were closely associated with mortality ($n = 66$) during follow-up (median 776 days; hazard ratio [$_{ln}$SN]: 4.63; 95% confidence interval: 1.93 to 11.11; $p = 0.001$ in multivariate analysis). SN reclassified patients to their correct risk strata on top of other predictors of mortality. In 155 patients with ventricular arrhythmia-induced cardiac arrest, SN levels were also associated with short-term mortality ($n = 51$; hazard ratio [$_{ln}$SN]: 3.33; 95% confidence interval: 1.83 to 6.05; $p < 0.001$ in multivariate analysis). Perfusing hearts with SN yielded markedly increased myocardial levels and SN internalized into cardiomyocytes by endocytosis. Intracellularly, SN reduced Ca^{2+}/calmodulin (CaM)-dependent protein kinase II δ (CaMKIIδ) activity via direct SN-CaM and SN-CaMKII binding and attenuated CaMKIIδ-dependent phosphorylation of the ryanodine receptor. SN also reduced sarcoplasmic reticulum Ca^{2+} leak, augmented sarcoplasmic reticulum Ca^{2+} content, increased the magnitude and kinetics of cardiomyocyte Ca^{2+} transients and contractions, and attenuated Ca^{2+} sparks and waves in HF cardiomyocytes.

CONCLUSIONS SN provided incremental prognostic information to established risk indices in acute HF and ventricular arrhythmia-induced cardiac arrest. (J Am Coll Cardiol 2015;65:339–51) © 2015 by the American College of Cardiology Foundation.

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ABBREVIATIONS AND ACRONYMS

AUC = area under the receiver-operating characteristic curve

BNP = B-type natriuretic peptide

CaM = calmodulin

CaMKIIδ = Ca^{2+}/calmodulin-dependent protein kinase II δ

CI = confidence interval

COPD = chronic obstructive pulmonary disease

CV = cardiovascular

CVD = cardiovascular disease

HF = heart failure

HR = hazard ratio

hs-TnT = high-sensitivity troponin T

ln = natural logarithm

NE = norepinephrine

NT-proBNP = N-terminal pro-B-type natriuretic peptide

OHCA-VF = out-of-hospital cardiac arrest with ventricular fibrillation

PLB = phospholamban

RyR2 = ryanodine receptor 2

SN = secretoneurin

Circulating biological substances (biomarkers) can identify specific pathophysiology in an individual patient (1). Cardiovascular (CV) biomarkers currently in clinical use are the B-type natriuretic peptides (BNPs), which reflect cardiomyocyte stress, and cardiac-specific troponins that measure cardiomyocyte injury (2). However, established biomarkers do not assess additional processes relevant to CV disease (CVD) progression such as increased neuroendocrine tone and cardiomyocyte Ca^{2+} homeostasis.

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Accumulating evidence suggests that the chromogranin-secretogranin (granin) family of proteins plays an important role in cardiac pathophysiology and may serve as CV biomarkers (3–8). One interesting member of this family is the high-capacity, low-affinity Ca^{2+}-binding protein secretogranin II (9). Secretogranin II is cleaved by the proteases PC1/3 and PC2 to the 33-amino acid peptide secretoneurin (SN) (SgII154-186) (9), and these proteases' activity is increased by 3-fold in the failing myocardium (10).

Previous work has shown that SN is the predominant form of secretogranin II immunoreactivity in plasma (11,12). By employing an SN-binding assay (epitope SgII154-165), we have demonstrated increased circulating SN levels in patients with heart failure (HF) (10). Additionally, SN has been shown to attenuate myocardial ischemia/reperfusion injury, reduce cardiomyocyte apoptosis, induce angiogenesis, and improve left ventricular function after myocardial infarction in experimental animal models (10,13). Given these data and the established role of granin proteins in modulating Ca^{2+} handling in noncardiac cells (14), we postulated that SN could have a direct, protective effect on cardiomyocyte Ca^{2+} handling. Accordingly, in this study we wanted to assess the potential of SN as a prognostic CV biomarker and explore the effects of SN on cardiomyocyte Ca^{2+} homeostasis.

METHODS

Details regarding methods and significant statistical and data elements are found in the Online Appendix.

CLINICAL COHORTS. We obtained blood samples <24 h following hospital admission in 143 patients with acute HF and <6 h following admission in 155 patients with ventricular arrhythmias and out-of-hospital cardiac arrest (153 patients with ventricular fibrillation; OHCA-VF). Blood samples were also available 24 h later in most patients (n = 104 for acute HF and n = 150 for OHCA-VF) and prior to hospital discharge in a subset of HF patients (n = 46). We additionally collected blood samples from 84 patients with acute chronic obstructive pulmonary disease (COPD) and from 62 healthy control subjects. Survival status was obtained from electronic hospital records.

EXPERIMENTAL MODELS. Internalization of SN (2.8 μM and 28 nM) was studied in neonatal mouse and rat cardiomyocytes and in isolated mouse hearts. For assessment of phosphorylation status, BayK (10 μM) was included in the perfusate of mouse hearts ex vivo to enhance myocardial Ca^{2+}/calmodulin (CaM)-dependent protein kinase II δ (CaMKIIδ) activity. Immunoblotting was performed with a primary antibody for SN (SgII172-186) (15). We assessed SN-CaMKIIδ and SN-CaM interactions by several methods, and examined CaMKIIδ activity in vitro by kinase activity assays. The effects of SN treatment on cardiomyocyte Ca^{2+} homeostasis were examined in isolated ventricular myocytes from adult healthy mice and in ventricular myocytes obtained adjacent to the infarct area (border zone) in HF rats (Online Table 1, Online Figure 1).

STATISTICAL ANALYSIS. Clinical data are expressed as mean ± SD with the exception of biomarkers, which are reported as median (quartile 1 to 3), and experimental data are presented as mean ± SEM. Differences between groups were examined by the Student *t* test or the Mann-Whitney *U* test, and serial measurements tested by the related-samples Wilcoxon signed rank test. Categorical data are presented as absolute numbers and percents, and were compared by the chi-square or the Fisher Exact test. We used Spearman rank correlation, and determinants of biomarker levels were assessed by linear regression analyses. Prognostic utility was assessed by Kaplan-Meier plots with SN quartiles compared by the log-rank test and by Cox

application filed by the University of Oslo regarding the use of secretoneurin as a biomarker in cardiovascular disease and in patients with critical illness. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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proportional hazards regression analysis. Biomarkers were transformed by the natural logarithm (ln) before regression analyses. Calibration of the basic risk model was examined by calculating the category-free net reclassification index (16), and prognostic accuracy was assessed by calculating the area under the receiver-operating characteristic curve (AUC); p values <0.05 were considered significant.

The clinical studies were approved by the Regional Ethics Committees and the experimental studies performed according to National Institutes of Health guidelines.

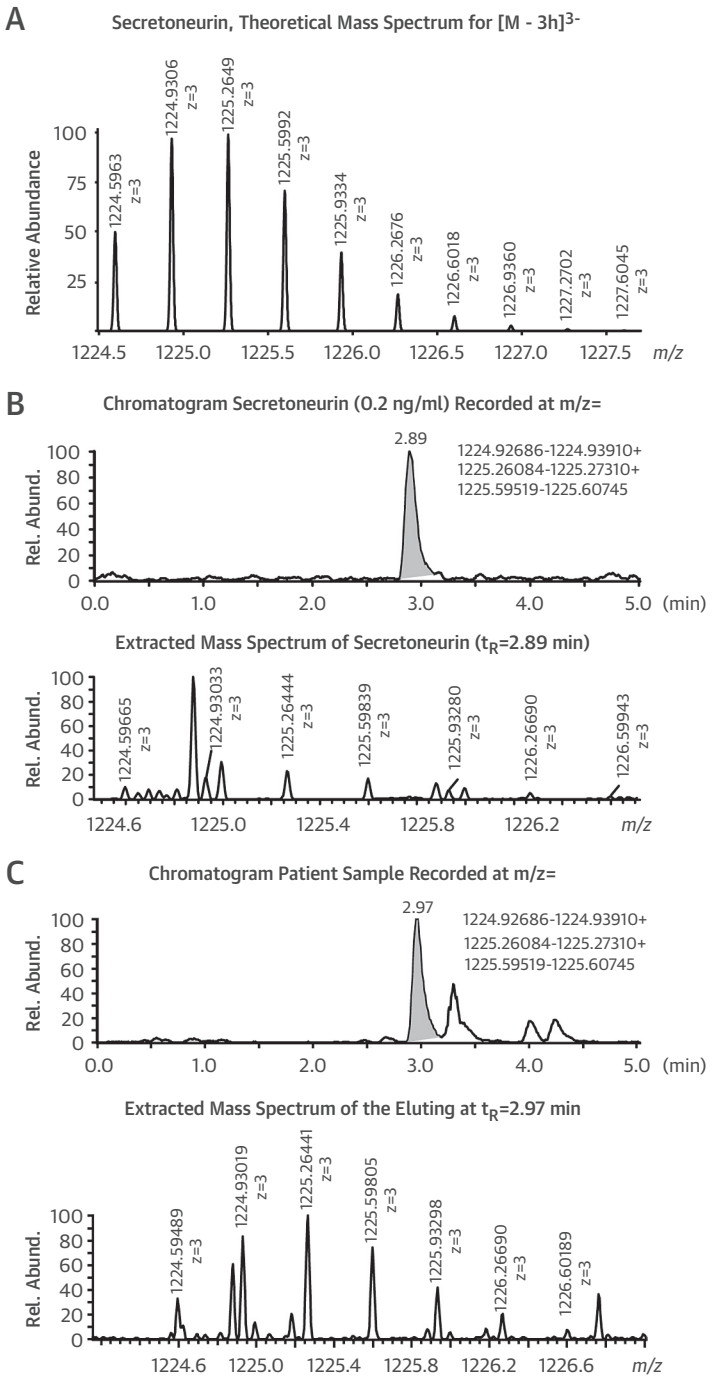
RESULTS

CIRCULATING SN LEVELS ON ADMISSION IN PATIENTS WITH ACUTE HF. We first confirmed the presence of free SN in the circulation of HF patients by liquid chromatography-mass spectrometry. The mass spectra of SN based on theoretical calculation (**Figure 1A**) and synthetic SN (**Figure 1B**) were found to be nearly identical to the spectrum in the plasma of HF patients (**Figure 1C**).

Using radioimmunoassay (17), we measured SN levels of 140 (122 to 170) pmol/l in acute HF patients with a range between 84 and 677 pmol/l (**Figure 2A**). SN levels correlated with N-terminal pro-B-type natriuretic peptide (NT-proBNP) ($r = 0.38$; $p < 0.001$) and high-sensitivity troponin T (hs-TnT) levels ($r = 0.27$; $p = 0.001$), but not with left ventricular ejection fraction ($r = -0.03$; $p = 0.77$) or norepinephrine (NE) levels ($r = 0.15$; $p = 0.08$). Patients classified as New York Heart Association functional class IV exhibited higher SN levels than the other patients (Online Figure 2). Creatinine clearance, diabetes mellitus, age, inhalation therapy with short-acting β_2-agonist, and NT-proBNP levels were associated with SN levels (details in Online Appendix). The duration of dyspnea prior to hospital admission (Online Figure 3) and the time from hospital admission to blood sampling did not influence SN levels in the acute HF patients ($r = -0.06$; $p = 0.46$).

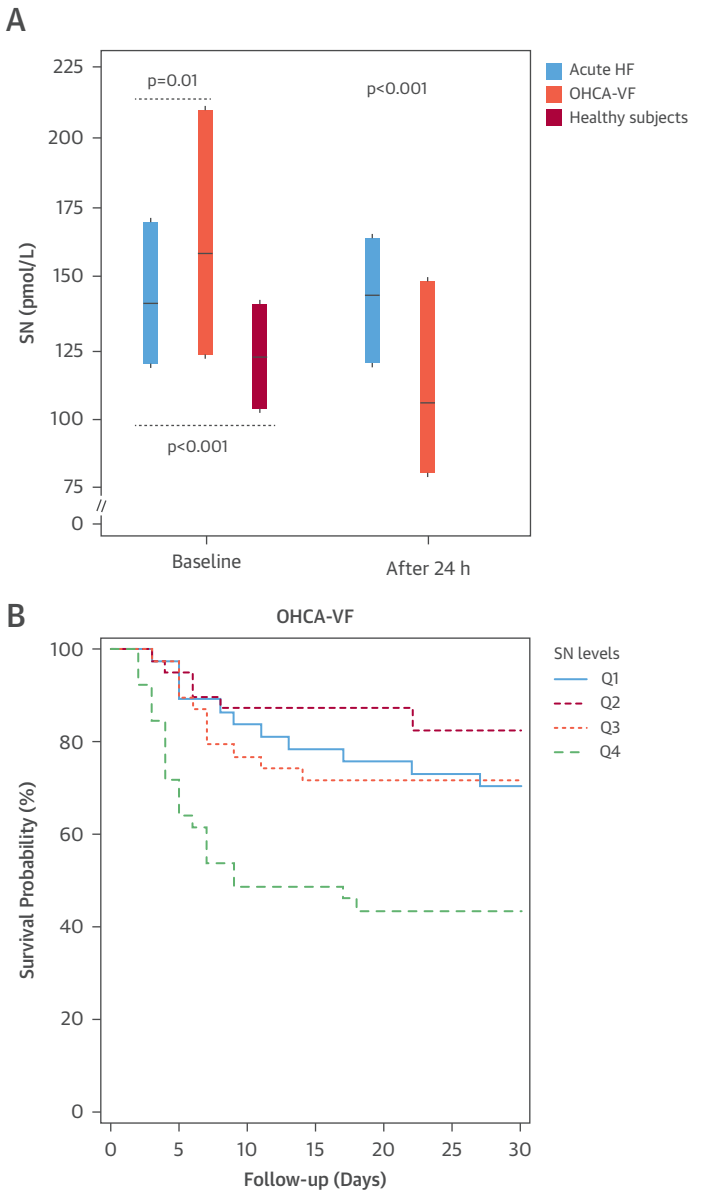
ADMISSION SN LEVELS ASSOCIATED WITH MORTALITY IN PATIENTS WITH ACUTE HF. Sixty-six patients (46%) died during a median follow-up of 776 (246 to 983) days (patient characteristics according to mortality in Online Table 2). There was a graded increase in mortality in proportion to admission SN levels (**Central Illustration**) ($p < 0.001$). SN was strongly associated with mortality by Cox regression analyses that adjusted for other risk variables, including NT-proBNP, hs-TnT, and NE levels (hazard ratio [HR] [$_{ln}$SN]: 4.63; 95% confidence interval [CI]: 1.93 to 11.11; $p = 0.001$) (**Table 1** and Online Table 3 with medication). Adding SN to a

FIGURE 1 SN Presence in HF Patients as Assessed by Liquid Chromatography-Mass Spectrometry



The mass spectrum of secretoneurin (SN) based on **(A)** theoretical calculation and the mass spectrum of **(B)** synthetic SN and **(C)** SN in heart failure (HF) patient samples were nearly identical.

FIGURE 2 SN Levels and Mortality



(A) In acute HF and out-of-hospital cardiac arrest with ventricular fibrillation (OHCA-VF) patients, secretoneurin (SN) levels were higher than in healthy subjects, with the highest level observed in OHCA-VF. After 24 h hospitalization, levels declined in OHCA-VF ($p < 0.001$) but remained elevated in acute heart failure (HF) patients. The **horizontal line** within the box represents the median concentration and the boundaries of the box quartiles (Q) 1 to 3. **(B)** Patients stratified according to SN quartiles on hospital admission in OHCA-VF ($n = 155$; $p < 0.001$ by the log-rank test).

model of clinical variables independently associated with mortality in our dataset, including NT-proBNP levels, reclassified a significant proportion of the patients to their correct risk strata (net reclassification index = 41%; $p = 0.01$). The effect by SN was primarily to lower the risk score in 69% of HF survivors (Online Figure 4A). SN discriminated between patients with a poor and a favorable prognosis in acute HF (AUC = 0.71 [95% CI: 0.63 to 0.78]); AUCs were lower for NT-proBNP (0.67 [95% CI: 0.59 to 0.75]) and hs-TnT levels (0.65 [95% CI: 0.57 to 0.73]).

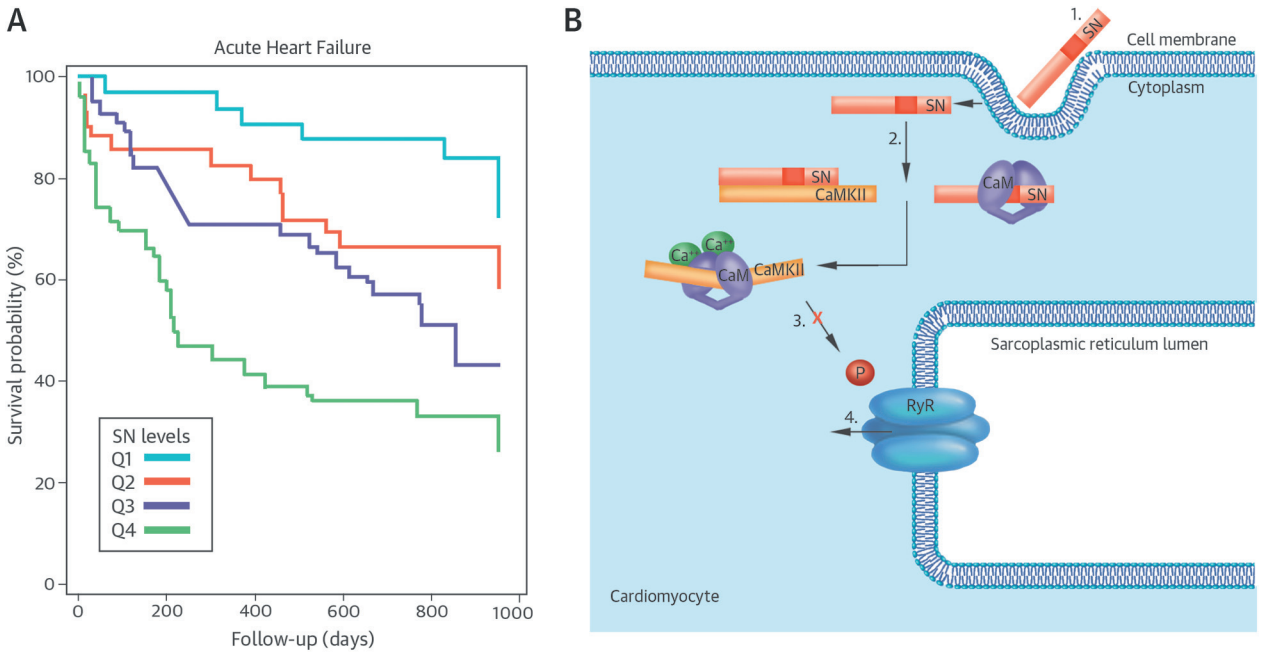
Circulating SN levels measured on admission for acute COPD were lower than admission levels in acute HF (median 136 [117 to 145] pmol/l; $p = 0.025$ vs. acute HF) and did not provide prognostic information for all-cause mortality (Online Figure 5). Of note, mortality rates and dyspnea severity were comparable between patients with acute COPD and acute HF (Online Table 4).

VENTRICULAR ARRHYTHMIA–INDUCED CARDIAC ARREST. SN levels provided prognostic information in acute HF but not acute COPD. As ventricular arrhythmias frequently are the immediate cause of death in HF patients, we also measured SN levels in an additional cohort of 155 patients with ventricular arrhythmia-induced cardiac arrest. In this cohort, median (quartile 1 to 3) SN level was 158 (122 to 209) pmol/l with values ranging from 58 to 2150 pmol/l (**Figure 2A**). SN levels correlated with time to return of spontaneous circulation ($r = 0.27$; $p = 0.001$) and estimated creatinine clearance ($r = -0.35$; $p < 0.001$), but not with cardiac biomarkers. Creatinine clearance, diabetes mellitus, and age were associated with SN levels after OHCA-VF.

In 62 healthy control subjects (38 male [61%], median age 58 [51 to 68] years), the median SN level was 121 (103 to 141) pmol/l (**Figure 2A**), which was lower than SN levels on admission for acute HF and OHCA-VF ($p < 0.001$ for both). Fifty-one patients (32%) hospitalized after successful resuscitation for OHCA-VF died within 30 days (characteristics according to mortality status in Online Table 5). Patients with SN levels in the fourth quartile had worse prognosis than patients with lower SN levels (**Figure 2B**) ($p < 0.001$). SN levels were also associated with short-term mortality in patients with OHCA-VF after adjusting for other risk factors (HR [$_{ln}$SN]: 3.33; 95% CI: 1.83 to 6.05): $p < 0.001$) (**Table 2**).

SERIAL SN LEVELS DURING HOSPITALIZATION FOR ACUTE HF AND OHCA-VF. Circulating SN levels in the first blood sample after admission for OHCA-VF were higher than baseline SN levels in acute HF patients ($p = 0.01$) (**Figure 2A**). However, although SN levels in OHCA-VF patients declined during the first 24 h ($p < 0.001$) (**Figure 2A**), no changes were observed during day 1 in the acute HF patients. Accordingly, SN levels were higher after 24 h in HF

CENTRAL ILLUSTRATION **Secretoneurin: Calcium Regulator and Biomarker**



Ottesen, A.H. et al. J Am Coll Cardiol. 2015; 65(4):339–51.

(A) As a biomarker, secretoneurin (SN) levels stratify mortality risk at hospital admission for patients with acute heart failure ($p < 0.001$ by the log-rank test for SN quartiles [Q]). **(B)** Experimental studies of SN in isolated cells and explanted hearts demonstrate that **(1.)** SN is internalized into cardiomyocytes and the intact heart by endocytosis. **(2.)** SN binds directly to calmodulin (CaM) and CaM-dependent protein kinase II δ (CaMKIIδ), and inhibits CaMKIIδ activity. **(3.)** This leads to reduced Ser2814-ryanodine receptor 2 (RyR2) phosphorylation, and **(4.)** improved Ca^{2+} homeostasis.

patients than in patients successfully resuscitated for OHCA-VF ($p < 0.001$) (Figure 2A).

In the subset of HF patients with serial biomarker measurements, the AUC for all-cause mortality for SN levels measured after 24 h was 0.65 (95% CI: 0.55 to 0.74) compared to 0.63 (95% CI: 0.53 to 0.72) for NT-proBNP, although the AUC on discharge for SN was 0.61 (95% CI: 0.45 to 0.74) and 0.56 (95% CI: 0.41 to 0.71) for NT-proBNP. Compared to concentrations at baseline, NT-proBNP levels tended to be lower after 24 h (median reduction 117 pg/ml; $p = 0.065$) and were significantly reduced at hospital discharge (median reduction 1172 pg/ml; $p < 0.001$). NE levels also were significantly reduced prior to hospital discharge (median reduction 1,596 pmol/l; $p = 0.001$). In contrast, SN levels stayed unchanged during the HF hospitalization with median 1.0 pmol/l increase during the first 24 h ($p = 0.91$) and median 1.0 pmol/l decrease on discharge ($p = 0.37$).

SN INTERNALIZED INTO CARDIOMYOCYTES AND THE INTACT HEART. On the basis of the strong association between SN levels and mortality in CVD and the association between granin proteins and Ca^{2+} homeostasis in noncardiac cells (14), we examined whether SN affects cardiomyocyte Ca^{2+} homeostasis. Although the receptor for SN has not been identified (9), a report has suggested that other short granin-derived peptides are internalized in cells by endocytosis (18), so we examined whether SN may be directly internalized into isolated cardiomyocytes and into the intact heart. We observed increased intracellular cardiomyocyte SN levels in proportion to the SN concentration in the suspension (Figure 3A). The internalization of SN was validated by confocal fluorescence microscopy (Figure 3B, Online Video 1). SN colocalized with Alexa 647-labeled transferrin (Invitrogen/Molecular Probes, Carlsbad, California) (Figure 3B), which is internalized via clathrin-mediated endocytosis (19) but not with dextran-alexa647, a marker of unspecific macropinocytosis (Online Video 1). SN also colocalized with markers of early (EEA1) and late endosomes (LAMP1) (Figure 3C).

TABLE 1 Predictors of Mortality During Follow-Up in Patients With Acute Heart Failure

	Hazard Ratio	95% CI	p Value
Univariate analysis			
Age, per 1-yr increase	1.04	1.02–1.07	0.002
Male	0.53	0.32–0.86	0.010
Body mass index, per 1 unit increase	0.94	0.89–0.99	0.012
Creatinine clearance, ml/min	0.98	0.97–0.99	<0.001
Heart rate, admission	1.00	0.99–1.01	0.40
Systolic blood pressure, admission, mm Hg	0.99	0.98–0.99	0.004
Diastolic blood pressure, admission, mm Hg	0.97	0.96–0.99	0.001
NYHA functional class IV versus II/III	2.01	1.23–3.29	0.006
LVEF, continuous	1.00	0.98–1.02	0.88
History of			
Heart failure	1.43	0.86–2.38	0.17
Myocardial infarction	1.00	0.62–1.63	1.00
PCI	0.69	0.37–1.29	0.24
CABG	1.17	0.66–2.05	0.59
Hypertension	0.83	0.51–1.36	0.47
Atrial fibrillation	1.07	0.66–1.74	0.78
Diabetes mellitus	1.78	1.08–2.95	0.024
Chronic obstructive pulmonary disease	1.85	1.14–3.01	0.013
$_{ln}$SN, admission*	5.06	2.83–9.03	<0.001
$_{ln}$NT-proBNP, admission*	1.53	1.24–1.89	<0.001
$_{ln}$hs-TnT, admission*	1.37	1.10–1.71	0.005
$_{ln}$NE, admission*	2.40	1.33–4.32	0.004
Multivariate analysis: final model			
Age, per 1-yr increase	1.06	1.03–1.09	<0.001
Systolic blood pressure, admission, mm Hg	0.99	0.98–0.99	0.001
Diabetes mellitus	1.77	1.02–3.09	0.044
Chronic obstructive pulmonary disease	2.20	1.29–3.76	0.001
$_{ln}$SN, admission*	4.63	1.93–11.11	0.001
$_{ln}$NT-proBNP, admission*	1.44	1.12–1.85	0.005
$_{ln}$NE, admission*	2.01	1.06–3.82	0.033

*Transformed by the natural logarithm (ln) before regression analysis.

CABG = coronary artery bypass grafting; CI = confidence interval; hs-TnT = high-sensitivity cardiac troponin T; LVEF = left ventricular ejection fraction; NE = norepinephrine; NT-proBNP = N-terminal pro-B-type naturietic peptide; NYHA = New York Heart Association; PCI = percutaneous coronary intervention; SN = secretoneurin.

TABLE 2 Predictors of Short-Term Mortality in Patients With Ventricular Arrhythmia–Induced Cardiac Arrest

	Hazard Ratio	95 % CI	p Value
Univariate analysis			
Age, per 1-yr increase	1.04	1.01–1.06	0.005
Male	1.33	0.57–3.13	0.51
Body mass index, per 1-U increase	1.02	0.96–1.08	0.59
Creatinine clearance, ml/min	0.99	0.98–0.99	0.025
History of			
Coronary artery disease	2.11	1.22–3.67	0.008
Diabetes mellitus	1.76	0.97–3.22	0.065
Hypertension	1.50	0.86–2.60	0.15
Heart failure	2.38	1.27–4.48	0.007
Witnessed cardiac arrest	0.32	0.15–0.69	0.004
Bystander CPR	0.90	0.51–1.60	0.72
Time to ROSC	1.05	1.03–1.07	<0.001
Therapeutic hypothermia	0.93	0.42–2.06	0.86
Coronary angiography	0.33	0.13–0.82	0.018
PCI	0.15	0.02–1.11	0.064
$_{ln}$SN, admission*	1.92	1.25–2.95	0.003
$_{ln}$NT-proBNP, admission*	1.39	1.12–1.73	0.003
$_{ln}$hs-TnT, admission*	1.25	1.02–1.55	0.034
Multivariate analysis: final model			
Age, per 1-yr increase	1.06	1.03–1.09	<0.001
Witnessed cardiac arrest	0.31	0.14–0.66	0.003
Time to ROSC	1.05	1.03–1.08	<0.001
$_{ln}$SN levels, admission*	3.33	1.83–6.05	<0.001

*Transformed by the natural logarithm before regression analysis.

CPR = cardiopulmonary resuscitation; ROSC = return of spontaneous circulation; other abbreviations as in Table 1.

We also observed a diffuse cytosolic SN signal that may reflect release of SN from endosomes. SN accumulated in the myocardium of isolated hearts perfused with SN (2.8 μM) (Figure 3D), indicating that SN internalization also occurs in the intact organ.

SN INHIBITS CAMKIIδ VIA DIRECT SN-CALMODULIN AND SN-CAMKIIδ INTERACTIONS. Bioinformatic analyses suggested that SN binds directly to both CaMKII and CaM (Figures 4A and 4B), which are nodal kinases that regulate intracellular Ca^{2+} handling in cardiomyocytes (20). In line with this, SN coprecipitated with CaMKIIδ in HEK293 cells overexpressing His-CaMKIIδ-T287D (Figure 4C). We also found a Ca^{2+}-dependent direct interaction between recombinant His-CaMKIIδ-T287D and SN by immunoprecipitation (Figure 4D) as well as a direct interaction between endogenous CaMKIIδ and SN in intact hearts perfused with SN (Online Figure 6A). A direct interaction between SN and CaMKIIδ was further demonstrated by surface plasmon resonance analyses, which revealed a dissociation equilibrium constant (K_D) for SN-CaMKIIδ of $8 \pm 3 \times 10^{-8}$ M, an association rate constant (k_a) of $3 \pm 1 \times 10^{3}$ $M^{-1}s^{-1}$, and a dissociation rate constant (k_d) of $(3 \pm 2) \times 10^{-4}$ s^{-1} (Figure 4E). This interaction was not present in the absence of Ca^{2+}. We further observed SN to bind CaM in a CaM-agarose pull-down experiment in the presence of Ca^{2+}. Precipitation was markedly attenuated in the presence of ethylene glycol tetraacetic acid, supporting a Ca^{2+}-dependence of the SN-CaM interaction (Figure 4F). Consistent with literature reports and validating the experimental conditions, CaM-agarose also precipitated CaMKIIδ in a Ca^{2+}-dependent fashion (Online Figure 6B). There was a direct SN-CaM interaction by surface plasmon resonance analyses with a K_D of $1.4 \pm 0.3 \times 10^{-7}$ M, a k_a of $1.3 \pm 0.1 \times 10^{3}$ $M^{-1}s^{-1}$, and a $k_d = 1.8 \pm 0.2 \times 10^{-4}$ s^{-1} (Figure 4G).

Using an in vitro kinase assay we observed that SN reduced CaMKIIδ activity in a dose-dependent

manner (**Figure 4H**). Although SN showed some sequence similarity to the region containing the Thr286 autophosphorylation site in CaMKII (Online Figure 6C), no phosphorylation of SN was observed (Online Figure 6D). We found no chelating effect of SN on Ca^{2+} (Online Figure 6E); thus, inhibition of CaMKIIδ activity by SN cannot be attributed to direct Ca^{2+} buffering.

SN REDUCES CAMKIIδ PHOSPHORYLATION. Autophosphorylation of CaMKIIδ at Thr286 leads to activation of the catalytic domain and a constitutively active kinase (21). Treating isolated cardiomyocytes with SN (2.8 μM) reduced CaMKIIδ autophosphorylation compared to control cells (**Figure 5A**). Similarly, in isolated mouse hearts perfused with BayK, inclusion of SN (2.8 μM) in the perfusate reduced myocardial CaMKIIδ autophosphorylation (**Figure 5B**).

The ryanodine receptor 2 (RyR2) is the major intracellular Ca^{2+} release channel in cardiomyocytes and its function is closely regulated by CaMKIIδ phosphorylation (20). We assessed RyR2 phosphorylation at Ser2814, an established CaMKIIδ regulatory site (20), in isolated BayK-perfused mice hearts in the presence and absence of SN. We observed a prominent BayK-induced increment in Ser2814-RyR2 phosphorylation, which was reversed by adding SN to the perfusate (**Figure 5C**). In contrast, Ser2808-RyR2 phosphorylation (**Figure 5D**), which is regulated by protein kinase A (22), was not influenced by BayK or SN treatment (**Figure 5D**).

We examined whether SN affects phosphorylation of phospholamban (PLB), a kinase that controls Ca^{2+} reuptake into the sarcoplasmic reticulum via the sarcoplasmic reticulum Ca^{2+} transport ATPase (20). Similar to observations made for RyR2, SN reduced CaMKIIδ-dependent phosphorylation of Thr17-PLB (**Figure 5E**), but did not influence protein kinase A-regulated Ser16-PLB phosphorylation (**Figure 5F**).

SN MODULATES CARDIOMYOCYTE CALCIUM HOMEOSTASIS. As SN regulates CaMKIIδ-dependent phosphorylation of RyR2 and PLB, we assessed SN effects on cardiomyocyte Ca^{2+} handling. In line with reduced CaMKIIδ activity at RyR2, SN (2.8 μM) reduced Ca^{2+} spark frequency in permeabilized and intact cardiomyocytes (**Figure 6A**). Using a 100-fold lower SN concentration (28 nM), which is only ~10-fold higher than circulating levels in patients and within the presumed myocardial SN concentration range after taking into account para- and autocrine production, SN induced a consistent and marked reduction in Ca^{2+} spark frequency (**Figure 6A**). SN treatment also reduced incidence of both Ca^{2+} sparks and waves in cardiomyocytes isolated from rats exhibiting HF following myocardial infarction (**Figure 6B**). SN treatment increased the magnitude of caffeine-elicited Ca^{2+} release, but did not affect rates of sarcoplasmic reticulum Ca^{2+} reuptake or sarcolemmal extrusion of Ca^{2+} (**Figure 6C**), thus linking the SN-induced increment in sarcoplasmic reticulum Ca^{2+} content to a reduction in RyR2 Ca^{2+} leak. Additionally, SN enhanced cardiomyocyte Ca^{2+} transient amplitude and reduced the time to half decay of the transient (**Figure 6D**), increased cardiomyocyte contraction amplitude, and reduced the time to peak of the contraction (**Figure 6E**).

DISCUSSION

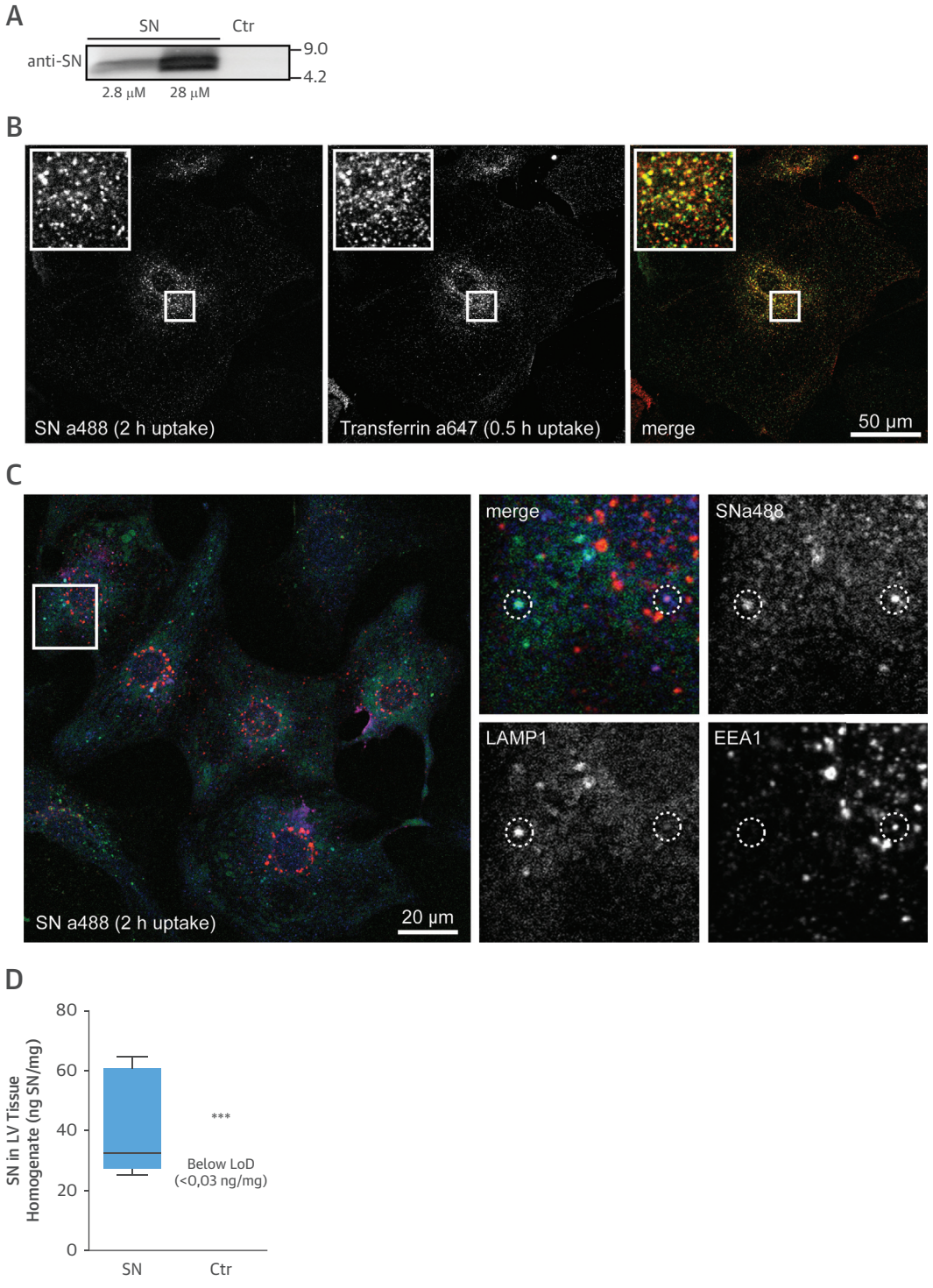
This study's principal results show that circulating SN levels provide strong prognostic information in patients with CVD and SN influences cardiomyocyte Ca^{2+} handling via direct CaMKIIδ inhibition (**Central Illustration**). Accordingly, SN seems to be a novel protective mediator activated in the most severely ill HF patients and a biomarker that reflects CV pathophysiology not covered by established risk indices.

A key requirement of novel biomarkers is that they add information beyond what is already available through established indices. In the present study, we demonstrate that SN provides incremental information to patient history, clinical assessment, and levels of hs-TnT, NT-proBNP, and NE in independent cohorts of patients with CVD. We chose to validate our results in a large cohort of OHCA-VF patients, as ventricular arrhythmias are responsible for a sizable proportion of HF deaths. The high SN levels measured within 6 h of OHCA-VF indicate patients with ventricular arrhythmias will have high circulating SN levels; those with more severe damage will have the highest levels. Of note, SN levels in healthy subjects were markedly lower than those in patients hospitalized for acute HF or after ventricular arrhythmia-induced cardiac arrest.

Accordingly, 3 SN characteristics seem to explain the powerful effect by SN on CV risk assessment: 1) circulating SN levels appear more narrowly distributed than most other circulating protein biomarkers (as found in the healthy subjects); 2) SN levels are strongly increased in the subgroup of patients with high risk of subsequent mortality; and 3) there are no close correlations between SN levels and other indices of risk, including hs-TnT, NT-proBNP, and NE levels.

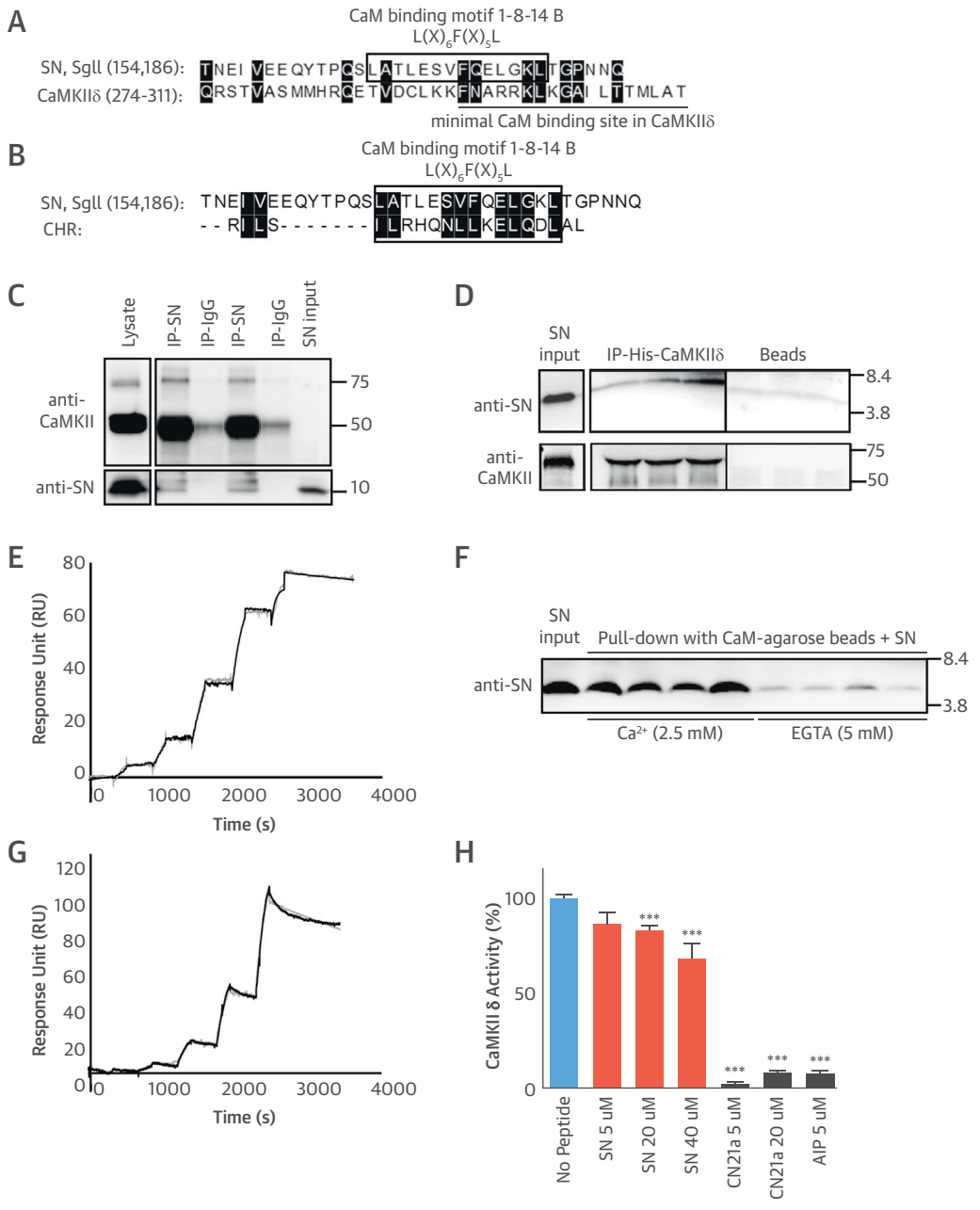
The relative contribution by different organs to circulating SN levels in CVD has not been fully established. However, current data support SN release to the circulation from the neuroendocrine system and from damaged cardiomyocytes and

FIGURE 3 SN Internalized Into Cardiomyocytes and the Intact Heart



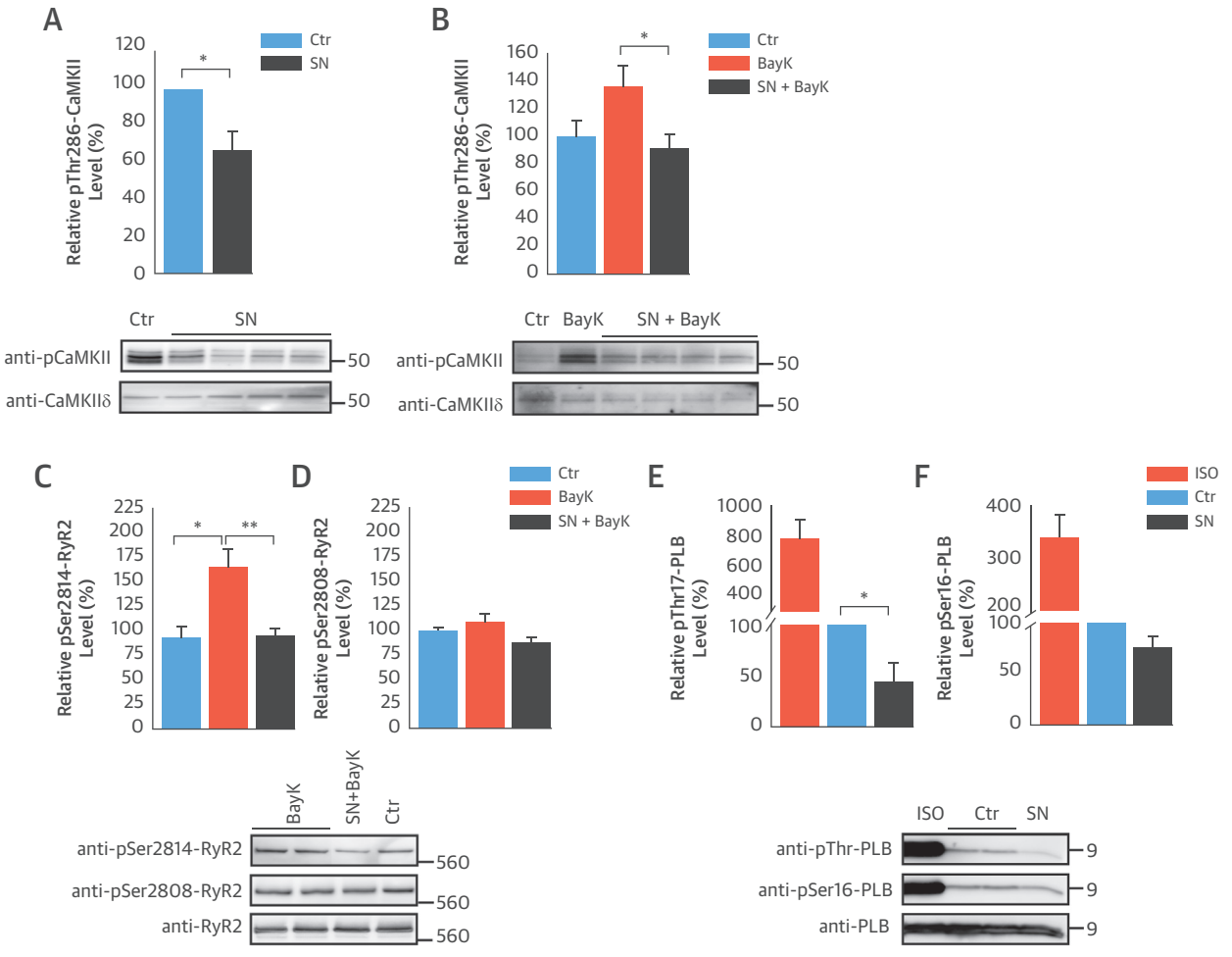
(A) Intracellular secretoneurin (SN) levels in neonatal mice cardiomyocytes increased in proportion to the extracellular SN concentration. **(B)** Confocal fluorescence microscopy identified Alexa-labeled SN as distributed in endosomes of neonatal rat cardiomyocytes **(left image; white)**. Transferrin is delivered to early endosomes **(middle image; white)**, and transferrin **(right image; red)** colocalized with SN **(right image; green, colocalization yellow)**. **(C)** Alexa-labeled SN **(green/white)** colocalized with early (EEA1 positive; **red/white**) and late (LAMP1 positive; **blue/white**) endosomes. **(D)** SN concentration was markedly increased in the myocardium of intact adult mice hearts perfused with SN. ***p $<$ 0.001 LoD = limit of detection; LV = left ventricular. (See Online Video 1.)

FIGURE 4 SN: Endogenous CaMKIIδ Inhibitor Via Direct SN-CaM and SN-CaMKII Binding



Secretoneurin (SN) exhibits sequence similarities to the CaM (calmodulin) binding motifs of **(A)** CaM-dependent protein kinase II δ (CaMKIIδ) and **(B)** chromofungin (CHR). **Black boxes** indicate identical or functionally similar amino acids (Lasergene, DNASTAR, Madison, Wisconsin). **(C)** SN immunoprecipitation demonstrated coprecipitation with CaMKII at 50 kDa and 72 kDa (HEK293 cell lysate). **(D)** Recombinant His-CaMKIIδ-T287D immunoprecipitation demonstrated coprecipitation with SN. Beads incubated without His-CaMKIIδ-T287D were used as negative control. **(E)** Titration binding profile for increasing concentration of biotin-SN and His-CaMKIIδ target. **(F)** CaM-agarose pull-down experiment demonstrated Ca^{2+}-dependent SN-CaM interaction (no binding with ethylene glycol tetraacetic acid [EGTA]). **(G)** Titration binding profile for increasing CaM concentration and SN target. The binding response **(black)** is overlaid with the fit of a 1:1 interaction model **(gray)**. **(H)** Effect of SN on CaMKIIδ activity (CN21a and autocamtide-2-related inhibitory peptide [AIP] were used as positive controls). ***$p < 0.001$.

FIGURE 5 SN Reduced CaMKIIδ Autophosphorylation and CaMKIIδ-Dependent Phosphorylation of RyR2 and PLB

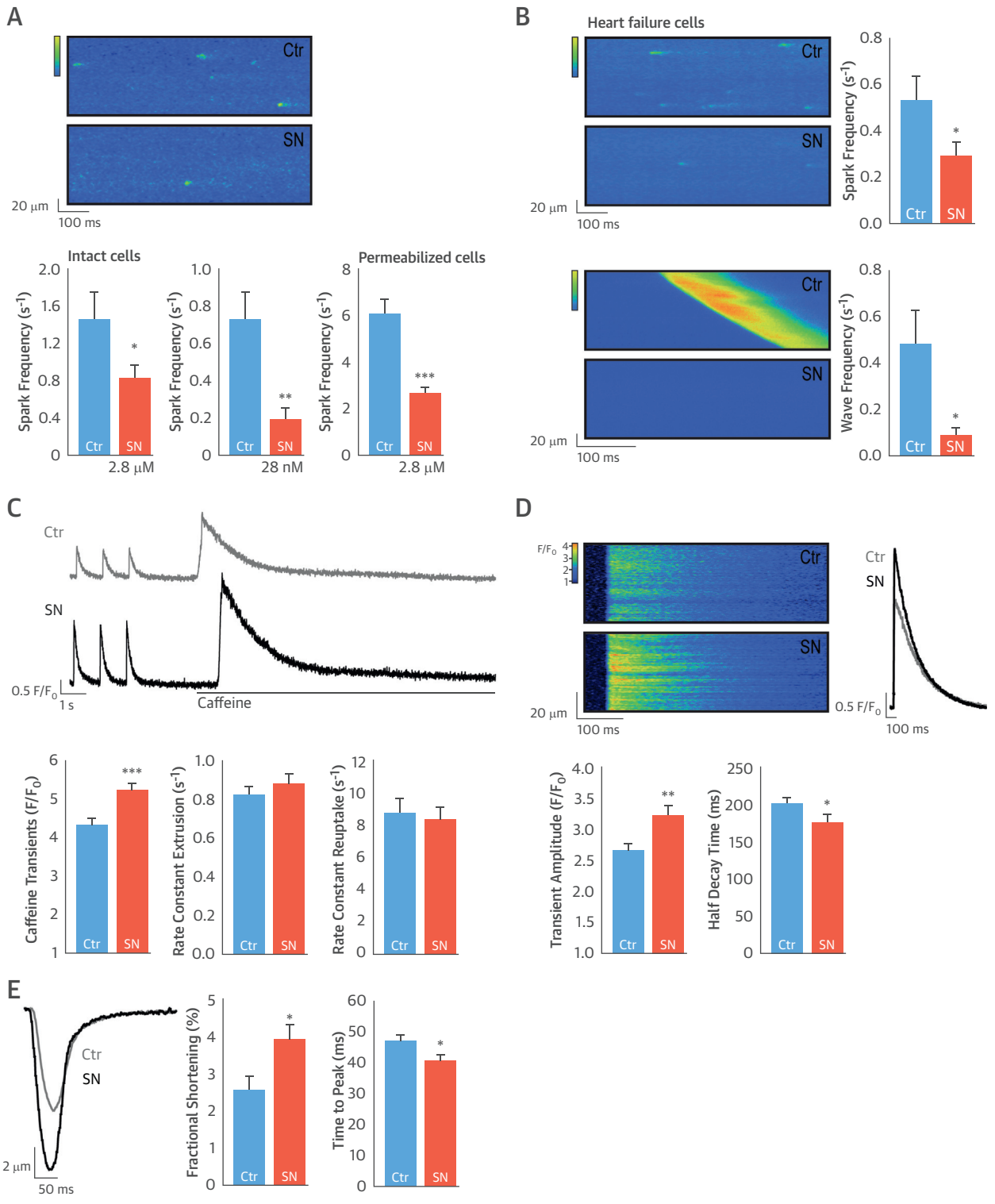


(A) Secretoneurin (SN) reduced autophosphorylation of Thr286-CaMKII in isolated neonatal cardiomyocytes. **(B)** SN reduced myocardial Thr286-CaMKII autophosphorylation in BayK-perfused hearts. **(C,D)** SN reduced myocardial BayK-induced Ser2814-RyR2 phosphorylation, but not Ser2808-RyR2 phosphorylation. **(E,F)** SN reduced CaMKII-dependent phosphorylation of Thr17-PLB in isolated neonatal cardiomyocytes, but demonstrated no effect on Ser16-PLB. ISO (100 nM) was used as a positive control. The lower panels are representative immunoblots. *$p < 0.05$; **$p < 0.01$. CaMKII = calmodulin-dependent protein kinase II δ; PLB = phospholamban; RyR2 = ryanodine receptor 2.

myocardium on the basis of increased SN immunoreactivity in the myocardium, but not in other organs (e.g., liver, spleen) of post-infarction HF mice (10). Neuroendocrine cells likely also contribute to circulating SN levels as granin proteins are found throughout the neuroendocrine system (9). SN levels were high within 6 h of OHCA-VF and normalized after cardioversion, which might support the release of SN from injured cardiomyocytes in situations of profound systemic and myocardial stress. Furthermore, as SN levels returned to normal within 24 h, SN seems to be a dynamic CV biomarker that reflects clinical outcome.

Additional support for the theory that SN is a biomarker specific for CVD is derived from the observed correlations between SN levels and levels of hs-TnT and NT-proBNP on admission for acute HF, and from the lack of prognostic value of SN levels in acute COPD. SN appears to be regulated by different mechanisms than established CV biomarkers, as elevated SN levels were observed throughout the acute HF admission, whereas NT-proBNP and NE levels decreased during hospitalization. Thus, SN provided a consistent and strong signal of pathophysiology of relevance for survival that was not modified by our HF patients' current

FIGURE 6 SN Modulates Cardiomyocyte Calcium Homeostasis



(A) Secretoneurin (SN; 2.8 μM and 28 nM) reduced Ca^{2+} spark frequency in intact cardiomyocytes. SN also decreased reduced Ca^{2+} spark frequency in permeabilized cardiomyocytes. **(B)** SN reduced Ca^{2+} spark and waves frequency in post-infarction heart failure rat cardiomyocytes. **(C)** Caffeine-elicited Ca^{2+} release demonstrated increased sarcoplasmic reticulum Ca^{2+} content during SN treatment but no change in rates of sarcoplasmic reticulum Ca^{2+} reuptake or Ca^{2+} extrusion from the cell. **(D)** SN increased Ca^{2+} transient amplitude and reduced the time to half decay. **(E)** SN enhanced mice cardiomyocyte contractions and reduced time to peak. Ctr = control.

therapy. Determination of the precise stimuli for SN production in HF will require additional study, but previous data have found that hypoxia, NE, and transforming growth factor-β stimulation increase SN production in cardiomyocytes (10,13). In contrast, as in our work here, previous data (23) also suggest that SN is not a surrogate marker for NE levels, nor a marker that is correlated with left ventricular ejection fraction. SN levels were higher in patients with impaired renal function, probably as a result of reduced clearance.

Earlier work has demonstrated beneficial effects of SN, including in vivo effects following myocardial infarction (13), but no direct receptor for SN has been identified (9,13). We now propose endocytosis, intracellular vesicle transport, and the release of SN into the cytosol as an alternative mechanism whereby SN may regulate cellular function. Endocytosis represents a mechanism permitting internalization of substances from interstitial fluid or plasma to distinct intracellular compartments (24). Internalization by endocytosis has also been reported for chromogranin A-derived peptides (18), suggesting that endocytosis could be an important mechanism whereby short granin peptides modulate cellular function.

We also present novel data that SN modifies cardiomyocyte Ca^{2+} handling by reducing CaMKIIδ activity via a direct interaction. Our data on the effects of SN on cardiomyocyte Ca^{2+} homeostasis support the theory that SN acts as a CaMKIIδ inhibitor, which has been found to reduce Ca^{2+} sparks while increasing sarcoplasmic reticulum Ca^{2+} content and the magnitude of Ca^{2+} transients and contractions (25,26). Whether SN may also reduce CaMKII activity due to increased oxidant stress (27) will require additional study. However, we demonstrate that SN reduces the frequency of Ca^{2+} sparks and waves in HF cardiomyocytes obtained from the border zone of the infarcted left ventricle, the area with the most pronounced oxidative stress (27).

Substances that are increased in the circulation of HF patients in proportion to disease severity may both promote disease progression or represent protective compensatory mechanisms. The observed actions of SN on cardiomyocyte Ca^{2+} homeostasis would be expected to be protective. Spontaneous RyR openings and resulting delayed afterdepolarizations are established mechanisms underlying triggered arrhythmia; excessive CaMKII-dependent sensitization of RyRs to Ca^{2+} is believed to contribute to increased arrhythmogenesis (20) and CaMKII inhibition protects against triggered arrhythmia (25). Hence, SN may be a compensatory mechanism during ventricular arrhythmias, a theory supported by the findings of high SN levels within 6 h of ventricular arrhythmia-induced cardiac arrest. HF is also widely reported to be associated with reduced sarcoplasmic reticulum Ca^{2+} content and release (28) and the larger Ca^{2+} transients and contractions observed during SN would be expected to counteract such deficits. Accordingly, SN is released by neuroendocrine and myocardial cells and reflects clinical outcome in CVD, whereas the direct effects of SN on CV pathophysiology may be protective by reducing diastolic Ca^{2+} leak via direct CaMKIIδ inhibition. This is analogous to high BNP levels being associated with poor prognosis, whereas the physiological effects of BNP are considered protective in CVD (2). The association between SN levels and cardiomyocyte Ca^{2+} homeostasis also may explain the potent prognostic information provided by SN in acute HF but not acute COPD, although we do not have access to cause of death in the HF patients. Alternatively, SN release from injured cardiomyocytes could have a local, negative effect via augmented CaMKIIδ activity and increased spontaneous Ca^{2+} release.

STUDY LIMITATIONS. The lack of information on the cause of death in our HF patients is a limitation of our study.

CONCLUSIONS

Circulating SN levels provide strong and complementary information to established risk indices in patients with acute HF and in patients with ventricular arrhythmia-induced cardiac arrest. We also demonstrated a direct effect by SN on cardiomyocyte Ca^{2+} handling via inhibition of CaMKIIδ activity, a key pathophysiological mediator in CVD.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Cardiovascular biomarkers such as B-type natriuretic peptides, which reflect cardiomyocyte stress, and cardiac-specific troponins, which reflect cardiomyocyte injury have diagnostic and prognostic value and are useful in guiding clinical management. Additional substances, such as secretoneurin (SN) may provide additional, complementary information proportionate to cardiovascular disease severity.

TRANSLATIONAL OUTLOOK: Additional clinical studies are needed to validate the incremental diagnostic value and therapeutic implications of novel biomarkers such as SN that reflect cardiomyocyte membrane permeability and may correlate with risk of progressive heart failure and ventricular arrhythmias.

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KEY WORDS biomarker, calcium cycling/excitation-contraction coupling, Ca^{2+}/calmodulin (CaM)-dependent protein kinase II, ventricular arrhythmias

APPENDIX For expanded Methods, Results, and References sections, and supplemental tables, figures, and a video, please see the online version of this article.