

**Title:** Plasma cBIN1 Score (CS) Identifies HFpEF and Can Predict Cardiac Hospitalization in Stable Ambulatory Patients

**Short Title:** Nikolova, CS as a blood based score of HFpEF patients

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## **ABBREVIATIONS:**

AUC – area under the curve

BIN1 – bridging integrator 1

BMI – body mass index

BNP – brain natriuretic peptide

cBIN1 – cardiac bridging integrator 1

CS – cBIN1 ScoreHF – heart failure

HFpEF – heart failure with preserved ejection fraction

HR – hazard ratio

IQR – interquartile range

LVEF – left ventricular ejection fraction

NT-proBNP - N-terminal prohormone of brain natriuretic peptide

NYHA – New York Heart Association

ROC – receiver operating characteristic

SD – standard deviation

## ABSTRACT

**Objective:** We determined, in stable ambulatory heart failure with preserved ejection fraction (HFpEF) subjects and matched controls, the capability of a novel blood based cardiac-specific cBIN1 Score (CS) to diagnose heart failure and prognosticate future hospitalization.

**Background:** Heart failure (HF) poses a costly health care burden worldwide with rising prevalence. Abnormal calcium signaling is intrinsic to HF pathophysiology and correlates with reduced expression of a cardiac membrane scaffolding protein, cardiac bridging integrator 1 (cBIN1). We hypothesize that CS, a numerical score derived from plasma cBIN1 concentration, is a diagnostic and prognostic biomarker of HF.

**Methods:** Plasma cBIN1 is quantified by an ELISA test, and CS is calculated as the natural log of the normalized reciprocal of plasma cBIN1 concentration. We determined CS among 52 clinically stable individuals with HFpEF (LVEF  $\geq 50\%$ ) (mean age  $57 \pm 15$  years old, 63% men) and 104 age and sex matched volunteers with no known history of HF. We obtained plasma concentrations of NT-proBNP, a marker of volume status, as comparison. Baseline co-morbidities and one year longitudinal clinical information were obtained through electronic medical records.

**Results:** Median CS is 0 (IQR -0.4 – 0.6) in the control cohort and is increased to 1.8 in the HFpEF cohort (IQR 1.5 – 2.3,  $p < 0.0001$ ). For HFpEF diagnosis, CS has a receiver operating characteristic (ROC) area under the curve (AUC) of 0.94 (95% CI 0.95 – 0.99) and NT-proBNP of 0.89 (95% CI 0.83 – 0.95). Kaplan-Meier analysis of one year cardiovascular hospitalizations reveals that HFpEF patients with CS  $\geq 1.8$  have a hazard ratio (HR) of 4.0 (95% CI 1.4 – 11.2,  $p=0.009$ ). Combining CS  $\geq 1.8$  with NT-proBNP  $\geq 300$  pg/mL, increases HR to 21.4 (95% CI 2.7 – 171.6,  $p=0.004$ ).

**Conclusions:** In a cohort of stable ambulatory HF patients with cardiomyopathies of multiple etiologies and preserved ejection fraction, a positive CS correlates with worsening myocardial health and predicts future hospitalization. CS, a marker of cardiac muscle health, provides a novel index to informing the management of stable ambulatory HF patients.

**KEY WORDS:** biomarkers; heart failure with preserved ejection fraction (HFpEF); cBIN1; cBIN1 Score (CS)

## INTRODUCTION

Heart failure (HF) is an ever-growing epidemic worldwide with high burden of both morbidity and mortality [1, 2]. Approximately 50% of this patient population has a phenotype characterized by a preserved left ventricular ejection fraction (LVEF) [1]. HF is a clinical diagnosis, which reflects the sequelae of diseased myocardium manifesting ultimately with familiar symptoms that include fluid retention, effort intolerance, and dyspnea. In our current clinical armamentarium, we have limited diagnostic tools to assess myocardial health. As surrogates, we rely on markers reflective of myocardial stretch as well as inflammatory, metabolic and fibrotic pathways, which are not specific to HF pathogenesis, but are affected in a broad array of conditions such as diabetes and hypertension.

The most validated biomarker family used in clinical practice is brain natriuretic peptide (BNP) and its N-terminal peptide (NT-proBNP), which increase in response to volume and pressure overload in the myocardium [3]. Albeit useful for the diagnosis of acute HF in patients presenting with dyspnea, approximately 30% of symptomatic heart failure with preserved ejection fraction (HFpEF) patients with chronic HF symptoms have normal BNP levels, despite elevation in pulmonary capillary wedge pressures. Such patients are less frequently prescribed needed therapy with diuretics for symptom relief [4, 5]. Furthermore, BNP metabolism is affected by adiposity, thus limiting its use in a patient population most in need of a HF marker, namely the obese [6-8]. In this single-center study, we explore a novel biomarker of myocardial health, the cardiac isoform of bridging integrator 1 (cBIN1). cBIN1 is a t-tubule membrane scaffolding protein in cardiomyocytes, which organizes the cardiac dyad-containing microdomains responsible for the initiation and regulation of myocardial calcium transients [9-13]. Loss of regulation in calcium signaling is a well described phenomenon in HF [14-16].

cBIN1 is transcriptionally decreased in heart tissue from end-stage HF patients as well as animal models of HF [10, 17]. Furthermore, cardiac origin cBIN1 circulates in blood making it accessible to assay from a venous blood sample [18]. In a previous cohort of arrhythmogenic cardiomyopathy patients, reduced plasma levels of BIN1 measured by a generic (not cardiac specific) test, correlated with HF stage and predicted future arrhythmias [19]. We recently cloned the cardiac isoform cBIN1 and found that it generates membrane microdomains within t-tubules [9]. These microdomains are turned over into blood as cBIN1-microparticles and cardiac origin cBIN1 concentration can be specifically assayed from plasma by ELISA [18]. In this study, we measured plasma cBIN1 concentration and computed the natural log of the normalized reciprocal of cBIN1 plasma concentration to create a cBIN1 score (CS), which provides a dimensionless index of muscle health and rises with worsening muscle failure. We tested the feasibility of CS as a diagnostic and prognostic marker in a single-center cohort of patients with confirmed diagnosis of HF on the background of heterogeneous underlying cardiomyopathies with preserved LVEF.

## METHODS

### *Study Design*

The study was approved by the Institutional Review Board (IRB) at Cedars-Sinai Medical Center (CSMC). Full informed consent was obtained from all subjects prior to participation in the study. The study involved two human populations including volunteers with no known history of HF and patients with known HFpEF.

All patients with clinical diagnosis of HFpEF (LVEF  $\geq$  50% and a history of HF) who were followed at the Cedars-Sinai Advanced Heart Disease clinic, were eligible for enrollment regardless of background cardiomyopathy. From July 2014 to November 2015, 52 patients with stable, chronic HF were enrolled in the study, and blood samples were obtained at the time of clinic visit. For each patient enrolled, detailed patient chart review was performed to characterize the HF cohort in terms of demographics, pertinent laboratory values, echocardiographic parameters and, if available, hemodynamic data per recent cardiac catheterization. NYHA classification of symptoms was assigned to each HF patient by the treating cardiologist at the index clinic visit. Additionally, chart review was performed every three months regarding the occurrence of significant clinical events in one year of follow-up. Outcomes of interest included any hospitalizations, cardiovascular-related hospitalizations, major adverse cardiac events (MACE) defined as myocardial infarction, revascularization for stable or unstable coronary or peripheral vascular symptoms, stroke or transient ischemic attack (TIA), unstable arrhythmias requiring hospitalization, ICD firing, death, heart transplantation or implantation of a ventricular assist device (VAD). The echocardiographic parameters obtained in the HF cohort followed the definitions set forward by the American Society of Echocardiography in their guidelines for chamber, left ventricular (LV) mass, and diastolic function quantification [20]. The

comparison cohort consisted of 104 age and sex matched volunteers without known cardiac diagnoses. Plasma from these subjects was obtained from Innovative Research with full informed consent. These volunteers completed a monitored health questionnaire at the time of consent regarding height, weight, age, ethnicity, coexisting comorbidities, and current medications. Each paired questionnaire and blood specimen collected were de-identified by Innovative Research. Sample processing and storage from the control cohort matched the protocol below.

*Cardiac hospitalization defined as the primary outcome during follow-up*

The cohort of 52 HFpEF patients underwent routine follow-up for the next one year at the Cedars-Sinai Heart Institute Advanced Heart Disease clinic. Cardiac hospitalization was predefined as any hospitalization after the time of cBIN1 blood draw, where the primary diagnosis was cardiac in origin. All chart review and adjudication were done by a two-physician panel who were not involved in the clinical care of the patients (AN and RMS).

*Sample Processing and CS Determination*

All plasma samples for the HF patients were collected at the time of clinic visit(s) and subsequently processed and stored in the Cedars-Sinai Medical Center Heart Institute Biobank per protocol. Whole venous blood was collected into lavender top (EDTA) tubes and stored immediately at 4°C for less than four hours. The plasma was separated from cells by spinning tubes in a refrigerated centrifuge at 2,250 g for 20 minutes. Then, over ice, 0.5 ml aliquots of plasma were pipetted into individual one milliliter de-identified, bar coded cryovials, flash frozen

with dry ice and ethanol, and stored in a -80°C freezer. Clinical data were obtained from chart review and stored in a de-identified Microsoft Access database, indexed by aliquot bar code.

The concentration of cBIN1 was determined using an assay provided by Sarcotein Diagnostics. In brief, a cBIN1 specific sandwich ELISA test was used, which employs a mouse monoclonal anti-BIN1 exon 17 as capture antibody (Sigma-Aldrich) and a HRP-conjugated detection recombinant antibody specific for exon 13 (Sarcotein Diagnostics). 96-well plates were coated with capture antibody, followed by loading with patient plasma samples. To detect the full plasma cBIN1 content from plasma microparticles, each plasma sample was subjected to osmotic shock to break up the microparticles by dilution with distilled water (3 volumes water, 1 volume plasma) before loaded to the ELISA plate [18]. Bound cBIN1 was detected using the HRP-conjugated anti-BIN1 antibody, with concentration determined from known protein standards. The ELISA reagents were purchased from BD Biosciences (BD OptEIA reagents kit, catalog 550534). Using positive control plasma samples with known cBIN1 concentrations, we validated that this assay is highly precise and reproducible with an inter-plate variability of < 5%.

CS is the natural log of the ratio of the median cBIN1 of controls (10ng/ml) to that of measured cBIN1:

$$CS = \ln\left(\frac{10}{[cBIN1]}\right)$$

We express our findings as CS, a normalized reciprocal, to be consistent with clinical convention of an elevated biomarker of HF to correlate with worsening cardiac status. Natural log of the ratio is used for CS because the distribution of the ratio is log normal.

### *NT-proBNP assay*

NT-proBNP values were obtained from the plasma of control and HFpEF patients. The Cedars-Sinai Medical Center clinical laboratory sends out the plasma samples to Quest Diagnostics Laboratory to perform the NT-proBNP assay using electrochemiluminescence. NT-proBNP was chosen over BNP due to its superior stability and higher mean recovery (residual immunoreactivity) when obtained from stored frozen plasma [21, 22]. Similarly, a natural log transformation of NT-proBNP was also performed and used for analyses.

### *Statistical Methods*

Data distributions were assessed for normality based on the quantile-quantile plot (QQ plot) and the Kolmogorov-Smirnov test. Continuous variables with a normal distribution were expressed and analyzed as means and standard deviations (SD) and compared using a two-sided Student's t-test. Continuous variables with non-normal distributions were analyzed with medians and interquartile ranges (IQR), and compared using the Mann-Whitney U-test. Categorical variables were compared using the chi-square test. Receiver operating characteristic (ROC) analysis was performed to determine the sensitivity and specificity of CS and NT-proBNP concentrations to diagnose HF. Since NT-proBNP has a non-normal distribution, log-transformations were used to assess the relationship with LV mass. CS and NT-proBNP were analyzed using Pearson's correlation analysis. Kaplan-Meier analysis was used to compare the differences in hospital-free survival rates between patients with high and low levels of CS using the log-rank test. Statistical analyses were conducted using SAS Version 9.3.1 software (SAS Institute Inc., Cary, NC) and RStudio Version 1.0.143 (RStudio, Inc., Boston, MA). Two-sided *p*-values were reported and a *p*-value <0.05 was considered statistically significant.

## RESULTS

### *Study cohorts*

The HFpEF cohort consists of 33 men (63%) with a mean age of  $57 \pm 15$  (mean  $\pm$  SD) years and a BMI of  $29 \pm 5.9$  kg/m<sup>2</sup> (**Table 1**). In the HFpEF cohort (**Table 2**), 75% of the patients suffer from NYHA Class II or greater degree of symptoms. Notably, 38% of the HF patients have normal diastolic parameters per echocardiography. The HFpEF patients exhibit excellent medical optimization of traditional cardiovascular risk factors as shown by well-controlled blood pressure and heart rate (displayed values are representative of the patient's known long term hemodynamic control), LDL cholesterol, hemoglobin A1c as well as non-smoking status. The most common class of medications used in this group is beta-adrenergic blocking agents, followed by angiotensin converting enzyme or receptor inhibitors, and diuretics. Most HFpEF (40/52) patients have normal renal function with mean estimated glomerular filtration rate (eGFR) of 90 mL/kg/min per the Cockcroft-Gault equation). Additionally, 104 volunteers were recruited for this study and matched for age and sex with the HFpEF subjects (**Table 1**).

### *CS is elevated in HFpEF patients*

The distribution of CS levels in the controls and HFpEF patients are seen in the violin plots of **Figure 1A**, indicating normal distribution of the values in both cohorts, with minimal overlap between the two groups. In the control cohort, CS has a median of 0.0 (open circle) with an interquartile range (IQR) of -0.4 to 0.6 (black boxed area). As compared to controls, CS levels are significantly higher in the HFpEF patients (**Figure 1**, right plot, median 1.8, IQR 1.5 to 2.3,

$p < 0.0001$ ). The mean CS in the HFpEF cohort ( $1.8 \pm 0.7$ ) is more than two standard deviations higher than the mean CS in the control cohort ( $0.1 \pm 0.8$ ).

In addition to CS, NT-proBNP, a well-established HF marker of hemodynamic load, was measured at the Cedars-Sinai clinical labs using the same frozen plasma samples for both cohorts. Natural log of NT-proBNP levels were calculated to aid with the comparison of the data sets. The distribution of ln NT-proBNP levels in the two studied cohorts is summarized in **Figure 1B**. In matched controls, median ln NT-proBNP level is 3.2 (IQR 2.7 - 4.1), which is increased to 5.6 (IQR 4.6 - 7.1) in the HFpEF group ( $p < 0.0001$ ). Distribution of ln NT-proBNP levels is positively skewed in both cohorts. An apparent large overlap of plasma NT-proBNP level distribution between the two cohorts is observed and occurs whether the scale is linear or logarithmic (**Supplemental Figure 1**).[23]

### *CS Diagnoses HF*

Since the CS level is higher in HFpEF patients, we proceeded to evaluate the performance of CS relative to NT-proBNP in identifying patients with HF. **Figure 1** juxtaposes the performance of the two biomarkers - CS (panel A) and ln NT-proBNP (panel B). Both the CS and NT-proBNP assays discriminate between the control and stable HFpEF cohorts; however with greater overlap in the two distributions with the NT-proBNP assay. Furthermore, in the 13 HF patients characterized by mild symptoms as determined by NYHA Class I, median CS levels were already increased to 1.7 which is close to the median of the HFpEF cohort (IQR 1.2 – 2.0). In contrast, in this subset of early asymptomatic HF patients, NT-proBNP concentrations were only modestly elevated and in some subjects within age and sex adjusted normal levels, with a median level in this group of 111 (IQR 60 – 233 pg/mL). To further assess the discriminatory

capability of CS to distinguish subjects with HF from those without, ROC curves were generated. The diagnosis of HF by CS yielded an area under the curve (AUC) of 0.94 (95% CI 0.91 – 0.98) while the AUC for NT-proBNP was 0.89 (95% CI 0.83 – 0.95). Furthermore, an additive effect was observed when the two tests were combined, which resulted in a significantly improved AUC of 0.98 (95% CI 0.83 – 0.95) when compared to either CS or NT-proBNP alone ( $p = 0.01$  and  $p = 0.003$  respectively) (**Figure 2**). Using a CS value of 1.8 as a diagnostic cutoff, which is the median value in the HFpEF cohort and is greater than two standard deviations above the mean value in the control cohort, CS detects HF patients with a positive predictive value (PPV) of 88% and negative predictive value (NPV) of 81% (arrow in **Figure 2**).

#### *In HFpEF patients, CS correlates with LV Mass*

cBIN1 is a ventricular cardiomyocyte t-tubule membrane scaffolding protein, which organizes membrane microdomains that regulate calcium transients and its levels are decreased in failing cardiomyocytes [13, 18]. A known pathophysiological hallmark of HF is the development of LV hypertrophy [15, 24, 25]. We analyze the relationship between CS and LV mass, as estimated by echocardiographic parameters per the most recent chamber quantification guidelines by the ASE [20]. In 26 HFpEF patients who had an echocardiogram within three months of study enrollment, CS levels were determined together with LV mass and LV geometry. We found concentric LV hypertrophy in the HFpEF cohort (LV mass  $220 \pm 92$  g, **Table 2**). CS has a significant positive correlation with increasing LV mass ( $r = 0.41$ ,  $p = 0.039$ ) (**Figure 3A**). In contrast, NT-proBNP does not significantly correlate with LV mass ( $r = 0.13$ ,  $p = 0.56$ ) (**Figure 3B**). The observed increase in CS with increasing LV mass suggests that cBIN1 decreases with pathological remodeling of the heart which is consistent with prior reports of the

cBIN1 role in t-tubule remodeling.[9, 13] These results provide confidence that the cBIN1 assay is not reflective of myocardial mass, but rather serves as an indicator of myocardial health and remodeling. Of note, CS did not correlate with either eGFR or BMI in the HFpEF (Pearson's  $r=0.0$   $p=0.99$ , and  $r=-0.05$   $p=0.74$ , respectively), further supporting the specificity of CS for myocardial health. For NT-proBNP, there was a strong inverse correlation with eGFR and a trend towards inverse correlation for BMI (Pearson's  $r=-0.67$   $p<0.001$ , and  $r=-0.25$   $p=0.08$ ).

#### *Elevated CS prognosticates cardiovascular hospitalization in HFpEF patients*

Next, we explored whether, in addition to its diagnostic value (**Figure 2**), CS can serve as a prognostic marker in predicting future clinical outcomes in patients with HFpEF. Using Kaplan-Meier curves, we explored whether, within the HFpEF group, patients with elevated CS values had worse outcomes as defined by cardiovascular events in the subsequent one year period. The high prevalence of morbidity and mortality among the 52 patients in the HFpEF cohort is illustrated by the following frequency of events within one year of follow-up: 27 patients had sustained a hospitalization for any event, 26 patients required hospitalization for cardiovascular related event, 5 patients suffered a MACE, 3 patients had an AICD discharge, 2 patients died from cardiovascular causes, 4 underwent a heart transplantation and 1 underwent LVAD implantation.

**Figure 4A** illustrates the outcomes for the HFpEF patients within one year of follow-up as stratified per CS level  $\geq 1.8$  versus  $< 1.8$ . Among patients with CS  $\geq 1.8$ , 55% underwent at least one hospitalization related to a cardiovascular event, as compared to 21% of the patients in the HFpEF group with CS  $< 1.8$  ( $p = 0.005$ , log-rank test). Given that cBIN1 and NT-proBNP reflect different aspects of HF pathophysiology (muscle health versus fluid status, respectively),

we tested whether prognosis is improved when CS is combined with the NT-proBNP test. The HFpEF cohort was stratified into four subgroups using CS cut-off of 1.8 and NT-proBNP cut-off of 300 pg/mL (the median levels of these biomarkers in the HFpEF cohort). It is evident that the sicker patients (those with high CS and high NT-proBNP) have a higher rate of at least one cardiovascular event (75% of the patients) than the relatively healthier patients who have a low CS and low NT-proBNP values (6%). Using CS alone, the hazard ratio (HR) for cardiovascular related hospitalizations of patients within 12 months of follow-up is 4.0 for those patients with  $CS \geq 1.8$  compared to those with  $CS < 1.8$  (95% CI 1.41 – 11.22,  $p = 0.009$ ). If NT-proBNP is used alone to prognosticate CV hospitalization, HR for NT-proBNP  $\geq 300$  pg/ml is 5.4 compared to NT-proBNP  $< 300$  pg/ml (CI 1.75 – 16.69,  $p = 0.0034$ ). However, using the above cut-off values, when combining the two biomarkers,  $CS \geq 1.8$  and NT-proBNP  $\geq 300$  pg/ml carried a high HR of 21.4 compared to  $CS < 1.8$  and NT-proBNP  $< 300$  pg/ml (95% CI 2.7 – 171.6,  $p = 0.004$ ).

## DISCUSSION

### *Current HF Assessment*

HF is a global epidemic for which there is a need for improved diagnostic and therapeutic approaches [1, 26]. Our current blood based diagnostic armamentarium is extremely limited and per the 2017 AHA/ACC HF guidelines, only natriuretic peptides carry a class I recommendation for use in clinical practice [26]. Even for natriuretic peptides, there are published concerns regarding clinical parameters which affect their sensitivity and specificity. For instance, the interaction of NT-proBNP in the obese patients is complex and highly important in the current demographic of HF patients. In the Atherosclerosis Risk in Communities (ARIC) Heart Failure Community Surveillance Study, of 10,000 patients studied, only 32% of the participants were normal weight, with the rest being overweight or obese [7]. It is well established from multiple large cohort studies that NT-proBNP has an inverse correlation with body mass index (BMI). Furthermore, many patients suffering from obesity have associated comorbidities such as obstructive sleep apnea, and pulmonary hypertension, which can lead to NT-proBNP elevation, thus decreasing its specificity for diagnosing HF. In PARADIGM-HF, natriuretic peptide levels were predictive of the primary outcome (the composite of death from cardiovascular causes or a first hospitalization for HF) except in the moderately/severely obese group where such an association was weak [27].

An additional interesting phenomenon has been reported now by post hoc analyses of two randomized clinical trials focused on HFpEF patients, namely the I-PRERSERVE and TOPCAT trials [28, 29]. Both analyses have shown a greater treatment benefit in patients with the lowest NT-proBNP levels. These subgroups of patients do include individuals whose natriuretic peptide levels fall within the normal range or are modestly elevated. This phenomenon suggests the

presence of a stage of HFpEF not captured by conventional tests that is still responsive to therapy with neurohormonal modulating agents. Conversely, there likely exist more advanced stages of HFpEF characterized by elevated natriuretic peptide levels which might show irreversible cardiac remodeling not amenable to therapy. We believe this demonstrates the need for a biomarker to capture these early stages of myocardial pathology that are not well detected by current diagnostic tests.

In recent years, there has been a surge in the introduction of novel biomarkers of HF [6, 30-36]. The variety of biomarkers is reflective of the diverse pathological domains of this syndrome such as myocardial fibrosis, increased collagen deposition, activation of alternative metabolic pathways and upregulation of pro-inflammatory cascades. Soluble ST2 (sST2), galectin-3 (Gal-3), high sensitivity Troponin T (hs TnT), and growth differentiating factor 15 (GDF-15) all have been explored. Clinical studies in which they have been characterized have primarily examined their prognostic value in HF. As such, there is a IIB level of evidence for use of sST2 and Gal-3 as additive risk stratification tools in chronic as well as acute HF.[26] Most new biomarkers do not originate from cardiomyocytes, and performance characteristics and specificity can be limited [6, 36]. A biomarker originating from cardiac muscle and correlating with cardiac health could potentially serve both diagnostic and prognostic roles, as well as management in stable ambulatory patients.

#### *cBIN1 based CS is a marker of biochemical health of cardiomyocytes*

T-tubule remodeling and the resultant abnormal calcium transients are central to the pathophysiology of HF [13, 17, 37-45]. Our groups and others have identified that t-tubule remodeling can be a consequence of reduced cBIN1 [9, 10]. cBIN1 is responsible for forming

the membrane microdomains which organize dyadic L-type calcium channel (LTCC) and ryanodine receptor (RyR) couplons [11, 12]. The integrity of cBIN1-microdomains is critical for synchronized calcium release and efficient excitation-contraction coupling. In HF, myocardial cBIN1 is transcriptionally downregulated [17] impairing cardiac contractility and promoting ventricular arrhythmias [9].

It was recently found that the cBIN1-organized microdomains at the cardiomyocyte t-tubule membrane are continuously turned over and released externally into blood [18]. The resultant cardiac origin cBIN1 in plasma can be measured by ELISA and is reduced in patients with HF [18]. In this study we introduce CS, a measure of failing muscle health, which is based on the log of the normalized reciprocal of ELISA measured cBIN1 concentration. An important performance characteristic of CS is its clear separation between individuals with HF and those without HF (**Figure 1**). In fact, the mean CS in the HFpEF cohort (1.8) is more than two standard deviations higher than the mean CS in the control cohort (0.0). Moreover, the ROC curves (**Figure 2**) for CS and NT-proBNP demonstrate the additive role of the two tests in the diagnosis of patients with HFpEF, reflective of their different pathophysiological role in HF. By enhancing the power of NT-proBNP in detecting HFpEF, a cBIN1-based blood test provides an additive value to existing diagnostic tests in this diverse and complex patient population.

In addition to the diagnostic value of CS, it is important to note that CS directly correlates with LV mass in the HFpEF cohort, and high CS levels predict worse clinical outcomes. Reports from animal models indicate that t-tubules widen in hypertrophied HFpEF hearts [46], similar to the phenomenon observed in systolic failure models of cBIN1 deficient hearts [9]. LV remodeling is likely a global organ level response to a cellular loss of t-tubule function. Systolic dysfunction has already been identified as a contributor to exercise intolerance in HFpEF

patients [47]. With cBIN1 deficiency and the consequent t-tubule abnormality, mammalian hearts present with diminished cardiac reserve and electrical instability, thus with increased incidence of both pump failure and ventricular arrhythmias [9]. This link between the cellular role of cBIN1 and overall cardiac function helps explain the value of CS in predicting cardiovascular related hospitalizations in HFpEF patients during a one year follow-up period (**Figure 4**).

Another notable feature of CS is that it does not correlate with BMI nor, in the HFpEF cohort, creatinine clearance. These factors have been found to influence NT-proBNP levels and thus, limit use of natriuretic peptides in patients with obesity or with kidney dysfunction, which constitute a large portion of the HF demographics. Thus, we believe that NT-proBNP and CS used together can enhance our diagnostic power in the HF domain by reflecting two different aspects of HF pathophysiology.

#### *CS is a potential biomarker of early stable HF*

The ideal diagnostic tool for HF will capture cellular remodeling prior to the onset of cardiac symptoms. Early diagnosis is even more challenging for HFpEF, given that a large proportion of HFpEF patients cannot be captured by the currently validated and available diagnostic tests, including echocardiographic parameters of diastolic dysfunction and NT-proBNP. For example, the investigators of the TOPCAT clinical trial characterize their HFpEF cohort and found that 33% of the participants had normal diastolic function by echocardiography and only 43% of the patients demonstrated LV hypertrophy [30, 48].

Similarly, in our current cohort, even with a demonstrated high rate of morbidity and mortality (**Figure 4**), more than half of the study subjects (27 patients) have NT-proBNP value of less than 300 pg/mL (a cut-off used in many clinical trials as an enrollment criterion for HFpEF patients) and 38% of the patients have normal diastolic function per echocardiography. In fact, all patients with NYHA Class I and II symptoms have NT-proBNP levels < 300 pg/mL and thus, would not be captured in many of the contemporary HFpEF clinical trials. In the same cohort, CS is already markedly increased in these patients with minor HF symptoms as classified by NYHA Class I and II. Thus, CS potentially could be able to detect early asymptomatic HF. CS has a normal distribution in the control cohort (**Figure 1**), suggesting that there are multiple stages of cellular remodeling occurring in a cohort that is yet to develop HF symptoms. Potential early detection by CS carries the promise of intervening at still reversible stages of the disease process, especially when the cardiomyopathy is caused by poorly controlled cardiovascular risk factors, such as obesity, hypertension or diabetes.

#### *Study Limitations and Future Directions*

Our study consists of a heterogeneous cohort of cardiomyopathies selected due to a documented HF diagnosis and preserved ejection fraction on echocardiography. We compared CS to a well-validated and established marker, NT-proBNP, in patients who are clinically stable. We are encouraged by the additive performance of the two tests, indicating different mechanistic origins: muscle health versus volume status, respectively. Future directions will assess cBIN1 in patients with reduced ejection fraction, as well as expand the single center study to multicenter national cohorts.

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## FIGURE LEGENDS

**Figure 1. Violin plots of cBIN1 score (CS) and ln NT-proBNP in the study cohorts.** Panel A shows the median CS values and IQR in the matched control group (median 0.0, IQR -0.4 – 0.6) and the HFpEF group (median 1.8, IQR 1.5 – 2.3). The figure shows clear separation between the two cohorts based on CS values with  $p < 0.0001$  per the Mann-Whitney U-test. Panel B contains median ln NT-proBNP values and IQR of the matched control (median 3.2 pg/mL, IQR 2.7 – 4.1) and HFpEF patients (median 5.6 pg/mL, IQR 4.6 – 7.1) with  $p < 0.0001$  per the Mann-Whitney U-test. The Panel B plot demonstrates substantial overlap between the two study groups.

**Figure 2. Receiver operating characteristic (ROC) curves for NT-proBNP, CS, and the two assays combined.** The area under the curve (AUC) for NT-proBNP is 0.89 (blue curve, 95% CI 0.83 – 0.95) and for CS is 0.94 (red curve, 95% CI 0.95 – 0.99). The AUC of the combined tests of NT-proBNP and CS is 0.98 (green curve, 95% CI 0.83 – 0.95), which shows that combining the two assays results in superior discriminating power between the control and heart failure cohorts as compared to NT-proBNP and CS alone ( $p = 0.003$  and  $p = 0.01$ , respectively). The arrow indicates a CS cut-off value of 1.8 which confers a positive predictive value (PPV) of 88% and negative predictive value (NPV) of 81% for the diagnosis of HF in the studied HFpEF cohort.

**Figure 3. CS and NT-proBNP correlation with left ventricular (LV) mass.** LV mass was estimated by echocardiography parameters per the cubed method. Panel A indicates that CS positively correlated with LV hypertrophy ( $r = 0.41$ ,  $p = 0.04$ , Pearson's correlation). Panel B

shows that no correlation is demonstrated between NT-proBNP and LV mass ( $r = 0.13$ ,  $p = 0.56$ , Pearson's correlation).

**Figure 4. Kaplan-Meier curves for cardiovascular (CV) hospitalization free survival.** Panel A displays the CV hospitalization free survival for the HFpEF patients using a CS cut-off value of 1.8, which is the median CS in this cohort (hazard ratio (HR) 3.98, 95% CI 1.41 – 11.22,  $p = 0.009$  for  $CS \geq 1.8$  versus  $CS < 1.8$ ). Panel B exhibits the Kaplan-Meier curves for the HFpEF patients using a combination of the CS and NT-proBNP assays with cut-off values of 1.8 and 300 pg/mL respectively. This panel suggests that prognostication of CV hospitalizations within 1 year of follow-up in the studied cohort can be strengthened by combining the two diagnostic tests ( $CS \geq 1.8$  and  $NT\text{-}proBNP \geq 300$  pg/ml was associated with a HR of 21.42 compared to  $CS < 1.8$  and  $NT\text{-}proBNP < 300$  pg/ml, 95% CI 2.68 – 171.60,  $p = 0.004$ ).

**Supplemental Figure 1. Violin plots for NT-proBNP in the HFpEF patients and matched controls.** The figure shows the median NT-proBNP level in the control cohort is 25 pg/mL with IQR 15 – 58 pg/mL, with higher levels observed in the HFpEF cohort (median 277 pg/mL, IQR 99 – 1264 pg/mL). While the levels in the two groups are statistically different with  $p < 0.0001$  per the Mann-Whitney U-test, the plots demonstrate the wide range of distribution of NT-proBNP values in the HF patients with considerable overlap with values observed in matched controls.

## TABLES

**Table 1. Baseline characteristics of HFpEF and matched controls**

**Table 2. CS distribution among subgroups**

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**Table 1. Baseline Characteristics of HFpEF and Matched Controls**

| Characterisitcs  | HFpEF (n=52) | Controls (n=104) | p-value |
|------------------|--------------|------------------|---------|
| Age (years)      | 57 ± 15*     | 54 ± 6.0         | NS      |
| Male (%)         | 33 (63)      | 66 (63)          | NS      |
| Race             |              |                  | NS      |
| White            | 30 (58)      | 58 (56)          |         |
| African-American | 10 (19)      | 21 (20)          |         |
| Hispanic         | 8 (15)       | 25 (24)          |         |
| Asian            | 4 (8)        | 0 (0)            |         |
| BMI (kg/m2)      | 29 ± 5.9     | 29 ± 5.5         | NS      |
| Hypertension (%) | 29 (57)      | 13 (10)          | <0.0001 |
| Diabetes (%)     | 5 (10)       | 9 (7)            | NS      |
| CKD (%)          | 5 (10)       | ---              | ---     |
| LVEF (%)         | 58 ± 7.1     | ---              | ---     |

HFpEF = heart failure with preserved ejection fraction; BMI = body mass index; CKD = chronic kidney disease; LVEF = left ventricular ejection fraction.

**Table 2. Baseline Characteristics of the HFpEF Cohort**

| Category                           | N        | Mean±SD          |
|------------------------------------|----------|------------------|
| <b>cBIN1 Score (CS)</b>            | 52 (100) | 1.8 (1.5 - 2.3)* |
| <b>NT-proBNP (pg/mL)</b>           | 50 (96)  | 277 (99 - 1264)* |
| <b>BNP (pg/mL)</b>                 | 45 (87)  | 184 ± 256        |
| <b>History of Smoking</b>          | 9 (17)   |                  |
| <b>NYHA Class</b>                  |          |                  |
| I                                  | 13 (25)  |                  |
| II                                 | 19 (37)  |                  |
| III                                | 17 (33)  |                  |
| IV                                 | 2 (4)    |                  |
| <b>Cardiomyopathy Type</b>         |          |                  |
| Infiltrative (sarcoid, amyloid)    | 5 (10)   |                  |
| HOcm                               | 5 (10)   |                  |
| Familial restrictive               | 3 (6)    |                  |
| Valvular                           | 6 (12)   |                  |
| Other                              | 33 (63)  |                  |
| <b>History of PAD/MI/PCI</b>       | 13 (29)  |                  |
| <b>History of arrhythmias</b>      | 29 (60)  |                  |
| <b>Presence of defibrillator</b>   | 15 (31)  |                  |
| <b>Echocardiography parameters</b> |          |                  |
| LVEF (%)                           | 52 (100) | 58 ± 7           |
| LVIDd (mm)                         | 48 (92)  | 48 ± 8.5         |
| LV Mass (g)                        | 48 (92)  | 220 ± 92         |
| <b>Diastolic Class</b>             |          |                  |
| Normal                             | 15 (38)  |                  |
| Class I                            | 11 (28)  |                  |
| Class II                           | 5 (13)   |                  |
| Class III                          | 8 (21)   |                  |
| <b>PASP (mmHg)</b>                 |          |                  |
| < 30                               | 20 (38)  |                  |
| ≥ 30                               | 18 (35)  |                  |
| <b>Laboratory Results</b>          |          |                  |
| Serum K (mEq/L)                    | 47 (90)  | 4.4 ± 0.4        |
| eGFR (mL/min)                      | 52 (100) | 90 ± 35          |
| LDL (mg/dL)                        | 36 (69)  | 100 ± 38         |
| Hemoglobin A1c (%)                 | 30 (58)  | 6 ± 0.9          |
| Hemoglobin (g/dL)                  | 41 (79)  | 13.4 ± 1.9       |
| <b>Hemodynamic parameters</b>      |          |                  |
| Systolic BP (mmHg)                 | 46 (69)  | 117 ± 16         |
| Diastolic BP (mmHg)                | 46 (69)  | 69 ± 10          |
| Heart rate (bpm)                   | 45 (68)  | 68 ± 11          |
| <b>Medication Therapy</b>          |          |                  |
| ACEI/ARB                           | 43 (89)  |                  |
| Diuretics                          | 33 (70)  |                  |
| Aldosterone antagonists            | 33 (69)  |                  |
| Nitrates                           | 20 (45)  |                  |
| Statin                             | 10 (23)  |                  |
| Anticoagulants                     | 17 (36)  |                  |
| Antiplatelet agents                | 34 (72)  |                  |

\*Median (IQR)

BMI – body mass index, HOcm – hypertrophic cardiomyopathy, PAD – peripheral arterial disease, MI – myocardial infarction, PCI – percutaneous coronary intervention, LVIDd – LV internal diameter in diastole, PASP – pulmonary arterial systolic pressure, K – potassium, GFR – glomerular filtration rate, LDL – low density lipoprotein, BP – blood pressure, ACEI – angiotensin converting enzyme inhibitor, ARB – angiotensin receptor blocker, SD – standard deviation

Figure 1

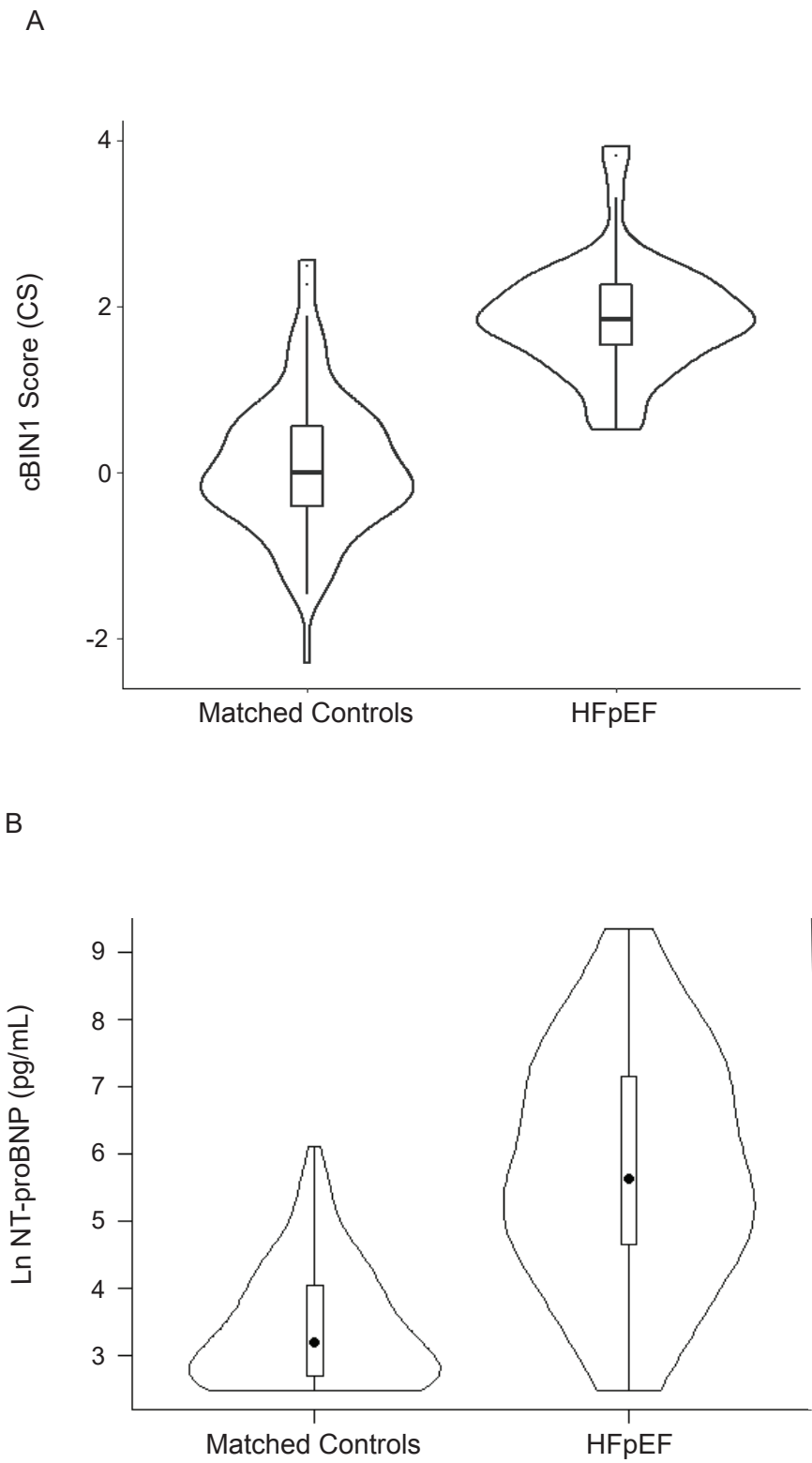


Figure 2

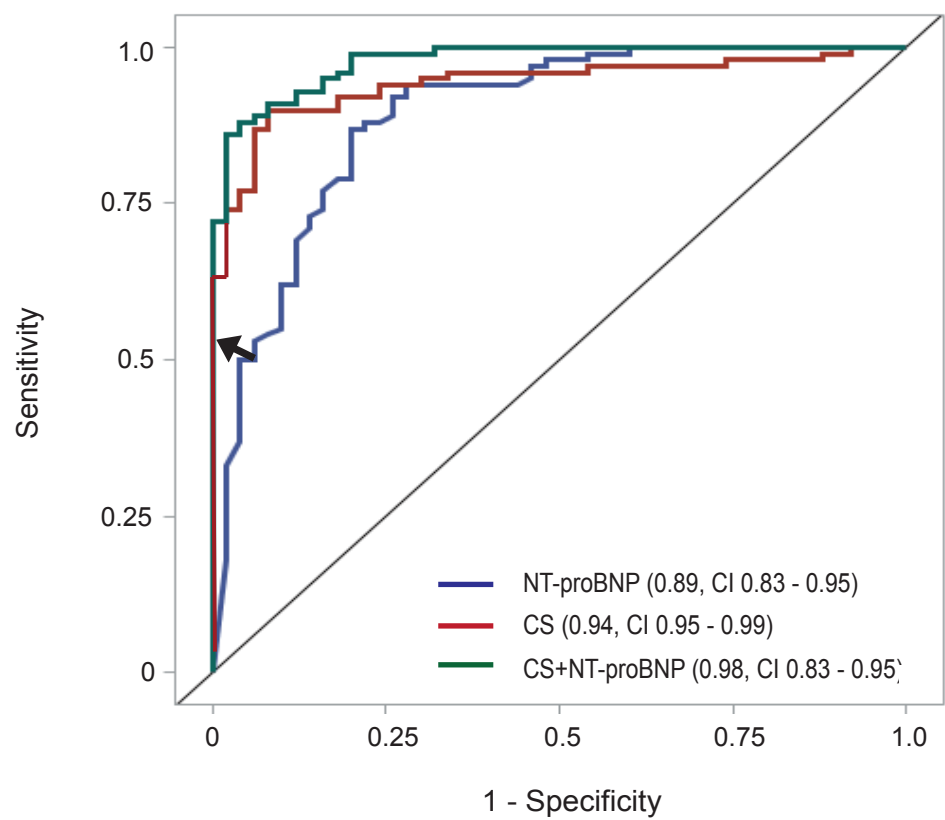
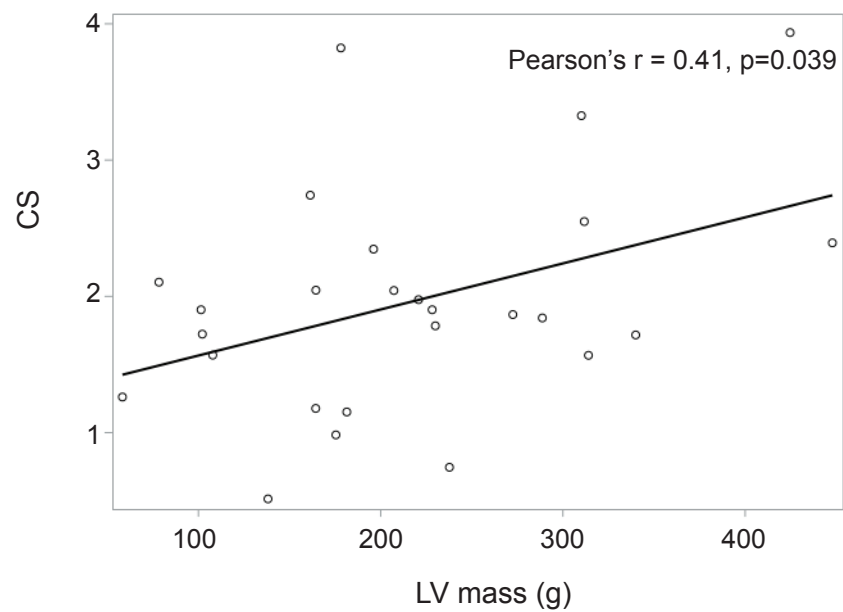


Figure 3

A



B

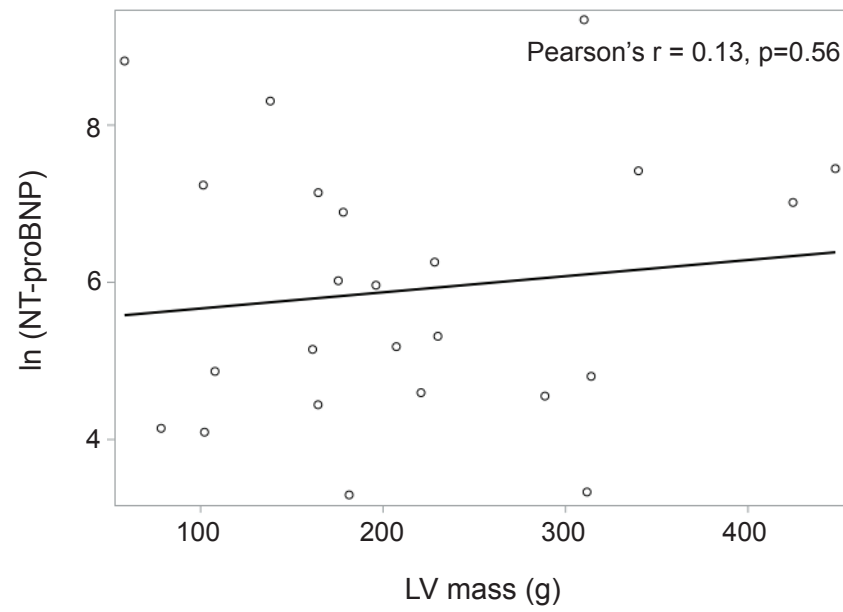
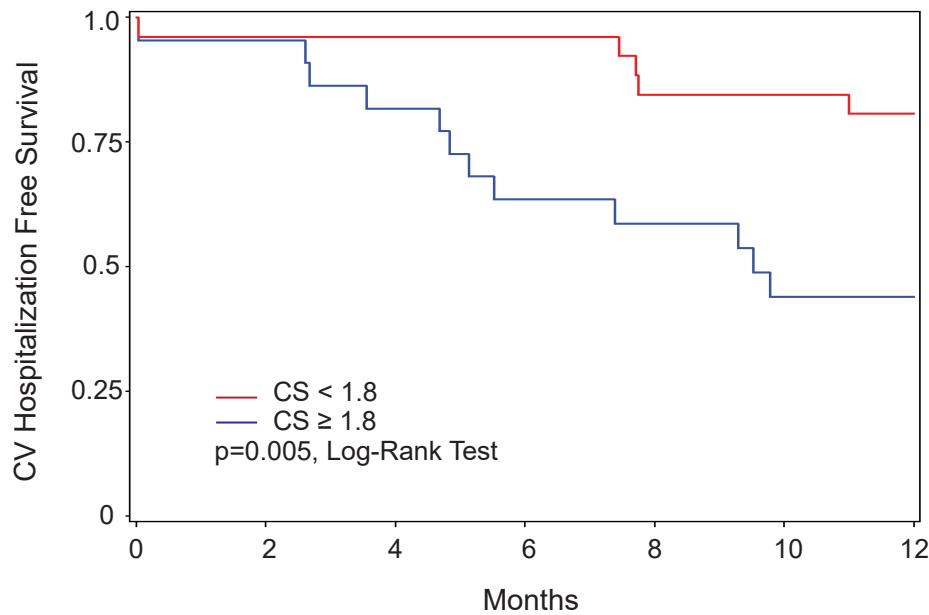


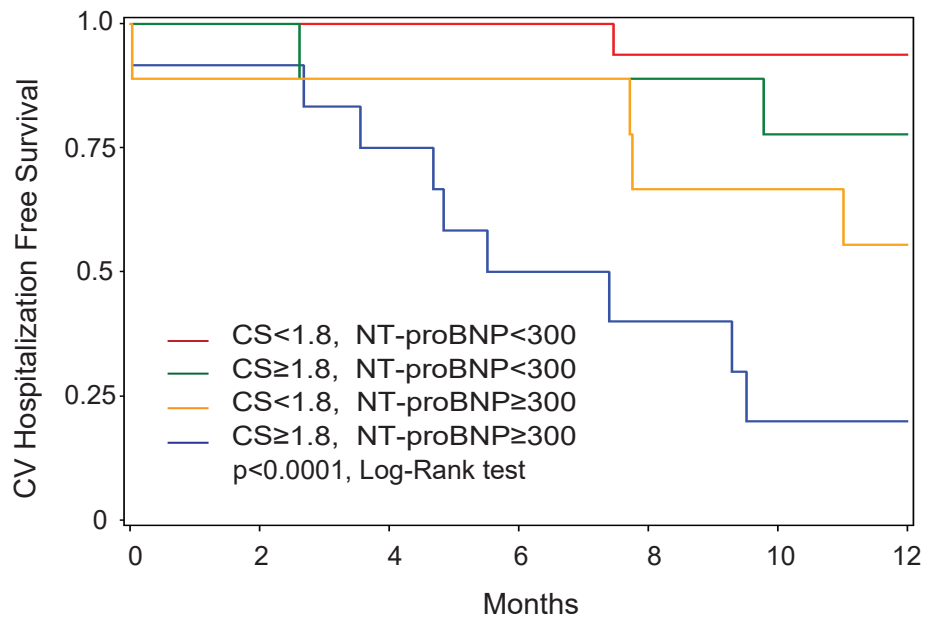
Figure 4

A



| Group | No. at risk |    |    |    |    |    |    |
|-------|-------------|----|----|----|----|----|----|
| —     | 24          | 23 | 23 | 23 | 20 | 19 | 19 |
| —     | 22          | 21 | 18 | 14 | 13 | 10 | 10 |

B



| Group | No. at risk |    |    |    |    |    |    |
|-------|-------------|----|----|----|----|----|----|
| —     | 16          | 16 | 16 | 16 | 15 | 15 | 15 |
| —     | 9           | 9  | 8  | 8  | 8  | 7  | 7  |
| —     | 9           | 8  | 8  | 8  | 6  | 6  | 5  |
| —     | 12          | 11 | 9  | 6  | 5  | 3  | 3  |

Supplemental Figure 1

