SUPPLEMENTAL MATERIAL

Prognostic value of elevated levels of plasma N-acetylneuraminic acid in heart failure patients

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Supplemental Methods

Enzyme-linked Immunosorbent Assay

The concentration of plasma neuraminidase was measured using a commercial kit (Ruixin Elisa Biotech Co., Ltd, Quanzhou, China) according to the manufacture's instruction.

Western Blot Analysis

Human cardiac tissues were frozen and stored at -80°C until analysis. The cardiac tissues were lysed in ice-cold lysis buffer and centrifuged at 12000g at 4°C for 20 min. After separation by 10% SDS-PAGE, total proteins were transferred onto polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany), which were blocked with 5% non-fat milk for 3 hours and incubated overnight with primary antibodies at 4°C, including anti-Neu1, anti-Neu2, anti-Neu3, anti-Neu4, anti-GAPDH (A6299, A8137, A13842, A5141, A19056, Abclonal), followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. An enhanced chemiluminescence system (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize the bands.

Histochemistry

Paraffin sections from human and mice cardiac tissues were deparaffinised with xylene and rehydrated with graded ethanol, and then we used anti-F4/80 (ab111101, abcam), anti-rabbit IgG-Cy3 (A22220, Jackson) and Fluorescein labelled Sambucus Nigra Lectin (FL-1301-2, Vectorlabs) to perform histochemistry. For nuclear staining, DAPI (C1006, Beyotime) was used. Tissues were imaged using confocal fluorescence

microscopy or panoramic slice scanner. Image-J was used to quantify fluorescence intensity after background correction.

Supplemental Figures and Figure legends

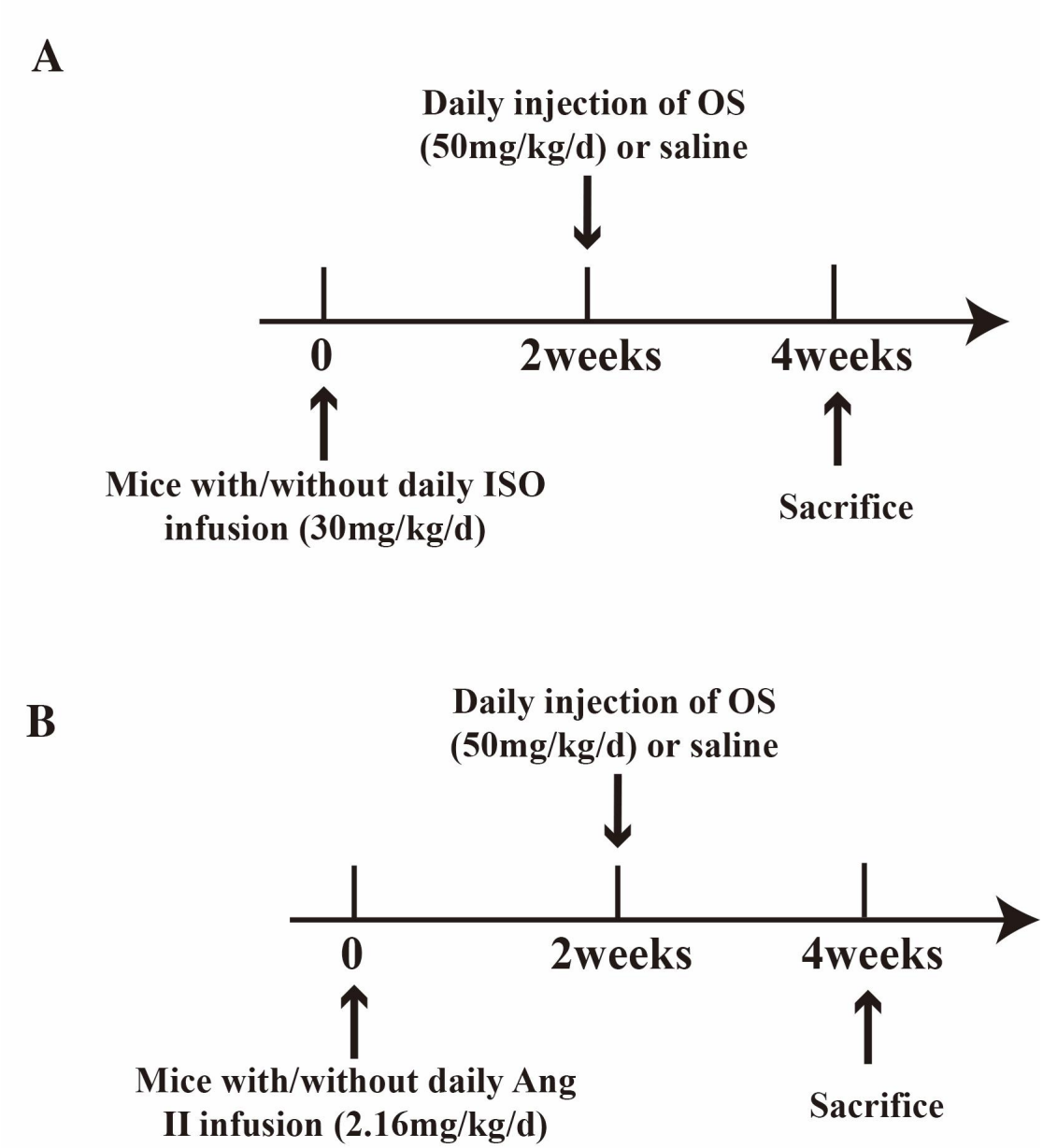

Figure I. The flowcharts of animal experiments. The mice were randomly assigned to groups with/without ISO (A) or Ang II (B) infusion for 4 weeks and were blindly treated with/without OS treatment for 2 weeks by an independent investigator. At 4 weeks, the examinations of mice were performed by another independent investigator. ISO, isoproterenol; Ang II, angiotensin II; OS, oseltamivir phosphate.

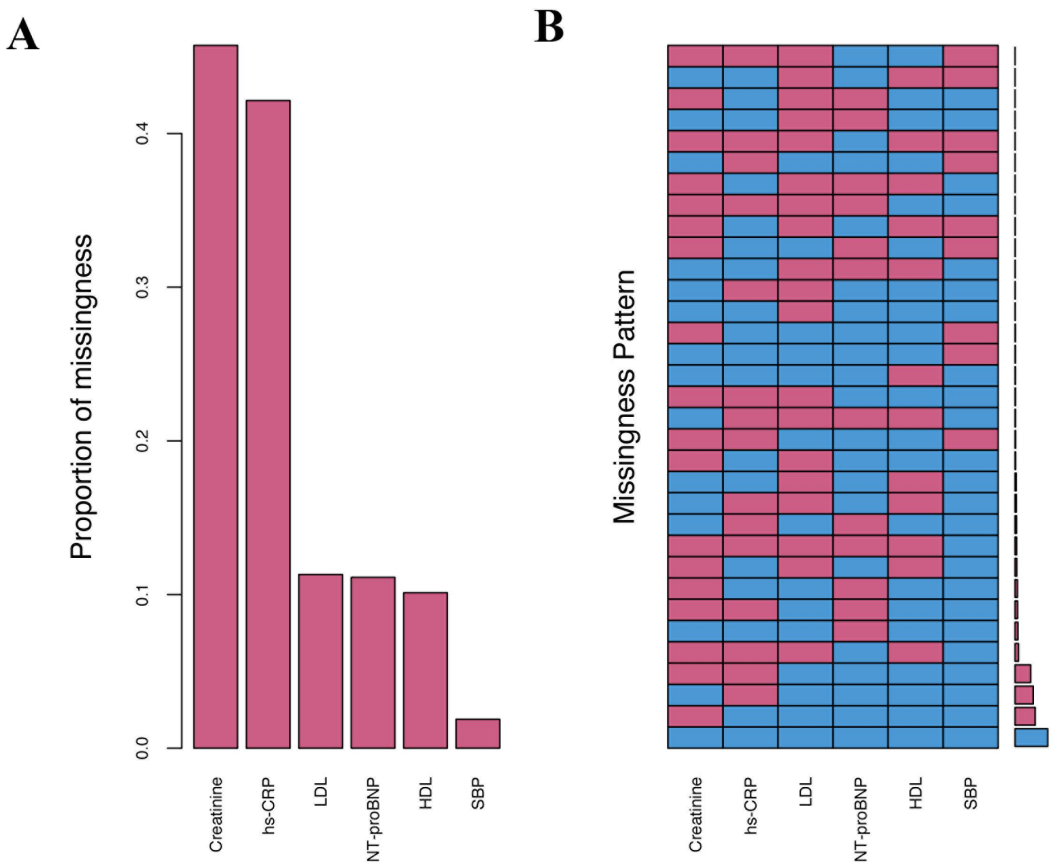

Figure II. **The missing variables used in the multivariable Cox regression model.**
A. Proportion of missingness values; B. Missingness pattern.
hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; HDL, high-density lipoprotein; SBP, Systolic Blood pressure.

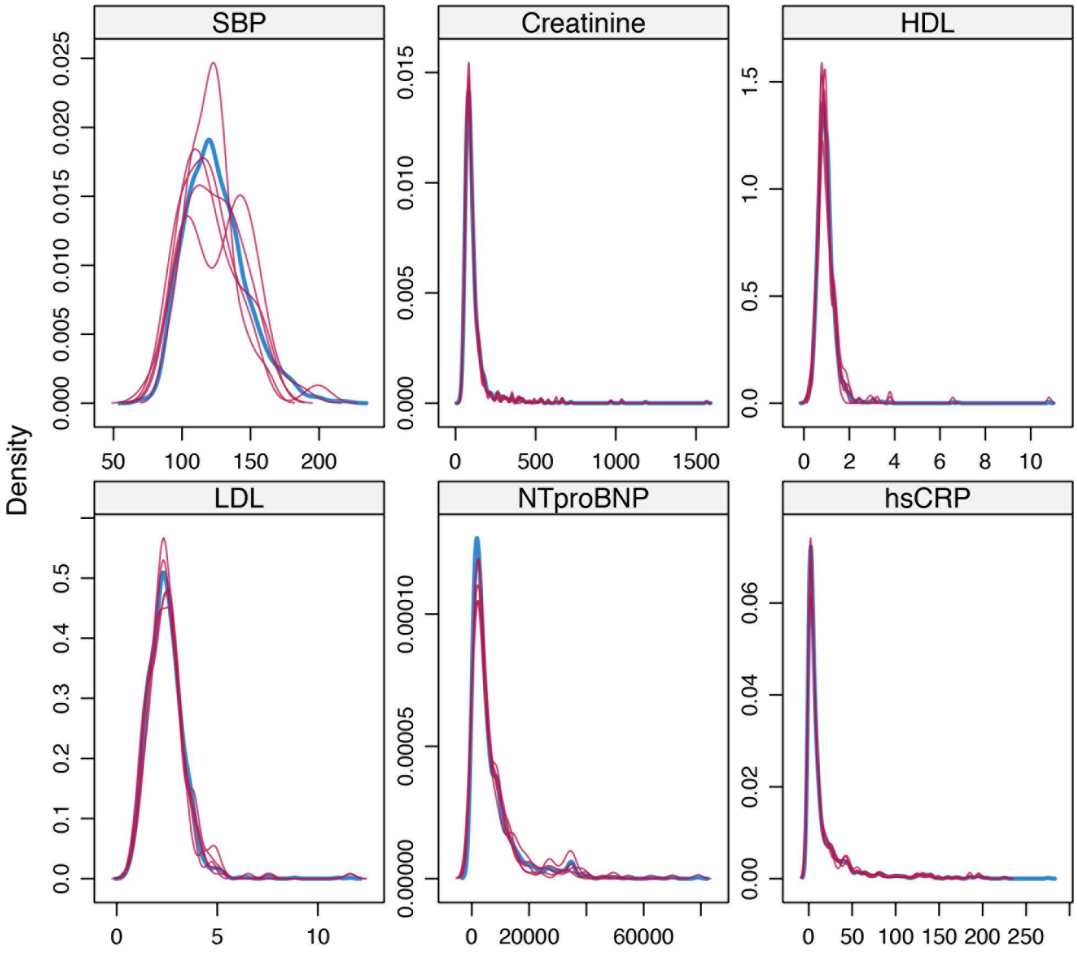

Figure III. The goodness of fit for missing data imputation illustrated by density plots. The blue curves are the observed variables and red ones are imputed variables. The similar distributions of observed variables and imputed variables suggest the excellent performance of missing data imputation.

hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; HDL, high-density lipoprotein; SBP, Systolic Blood pressure.

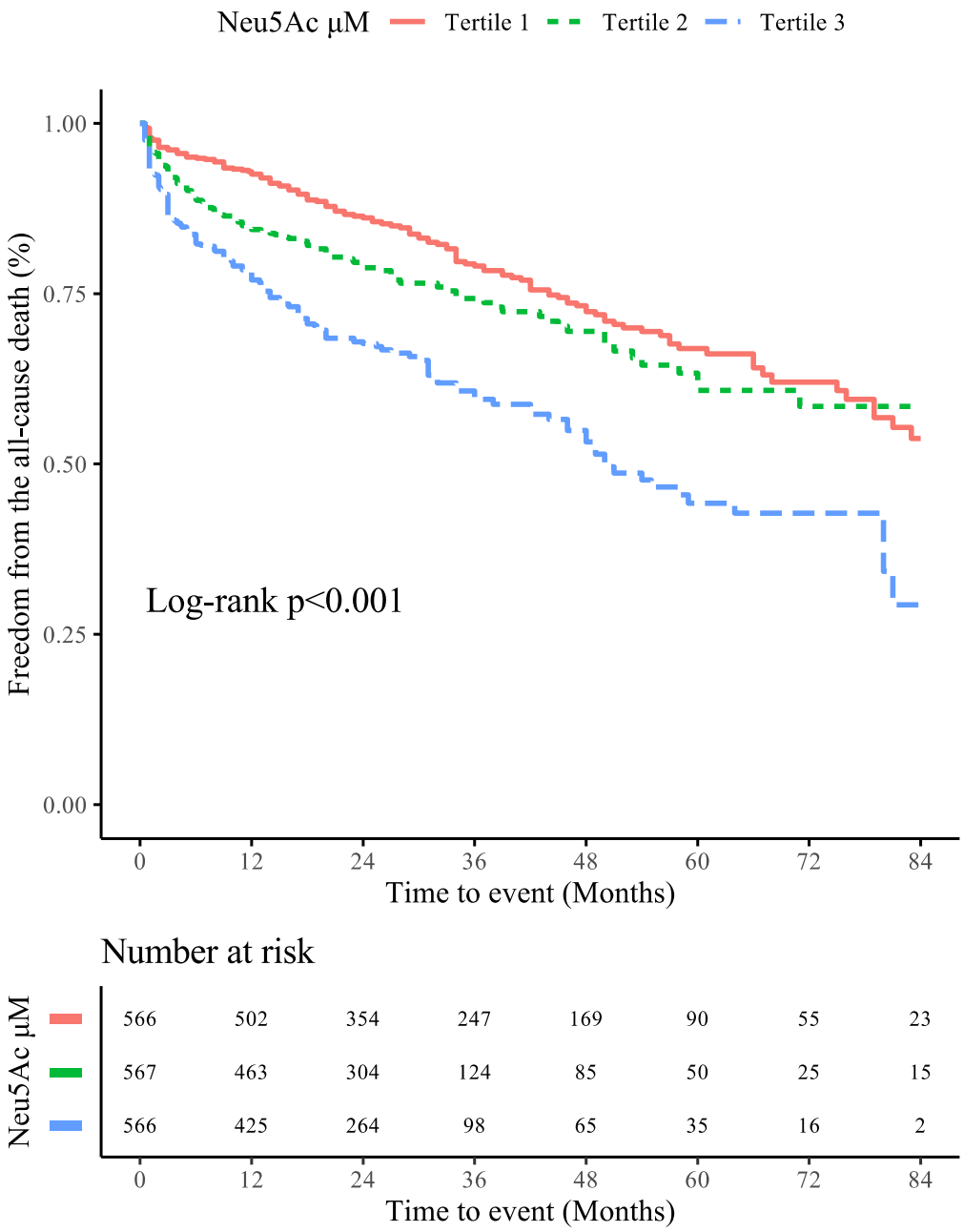

Figure IV. Kaplan-Meier curves of all-cause death associated with plasma Neu5Ac levels in the study cohort. Neu5Ac, N-acetylneuraminic acid. The p value was calculated using the log-rank test.

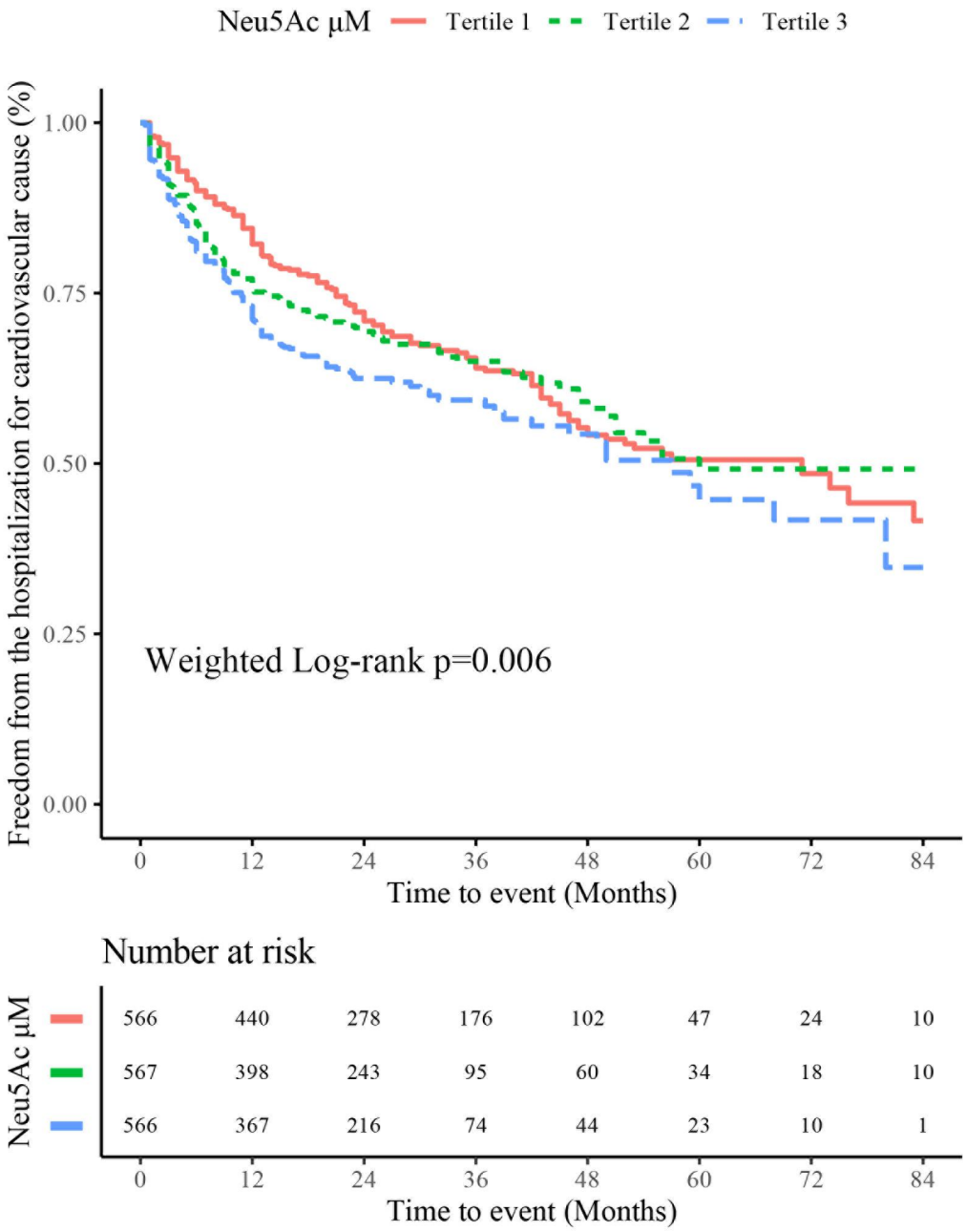

Figure V. Kaplan-Meier curves of cardiovascular rehospitalization associated with plasma Neu5Ac levels in the study cohort. Neu5Ac, N-acetylneuraminic acid. The p value was calculated using the weighted log-rank test (Fleming-Harrington) for non-proportional hazards.

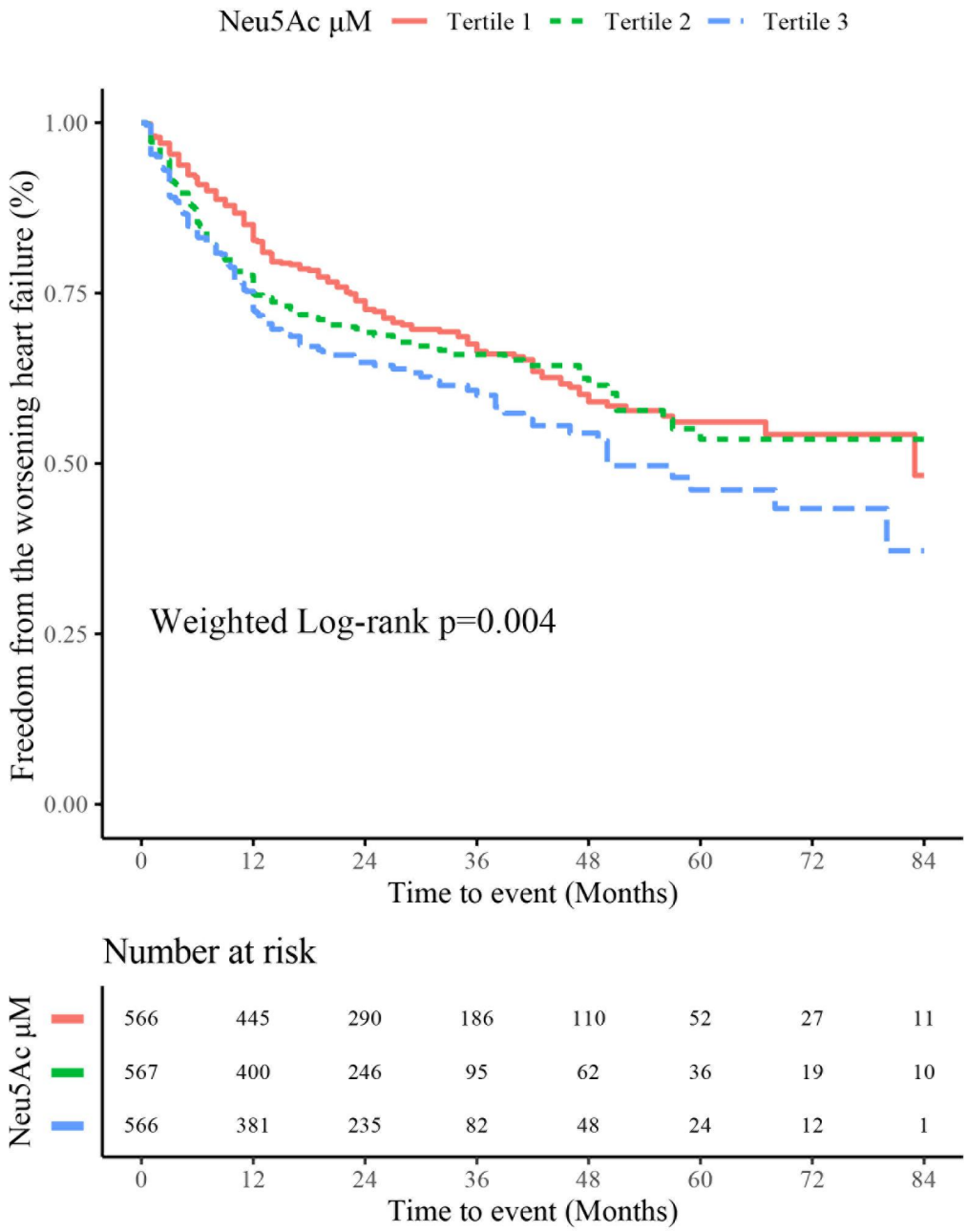

Figure VI. Kaplan-Meier curves of recurrence of heart failure associated with plasma Neu5Ac levels in the study cohort. Neu5Ac, N-acetylneuraminic acid. The p value was calculated using the weighted log-rank test (Fleming-Harrington) for non-proportional hazards.

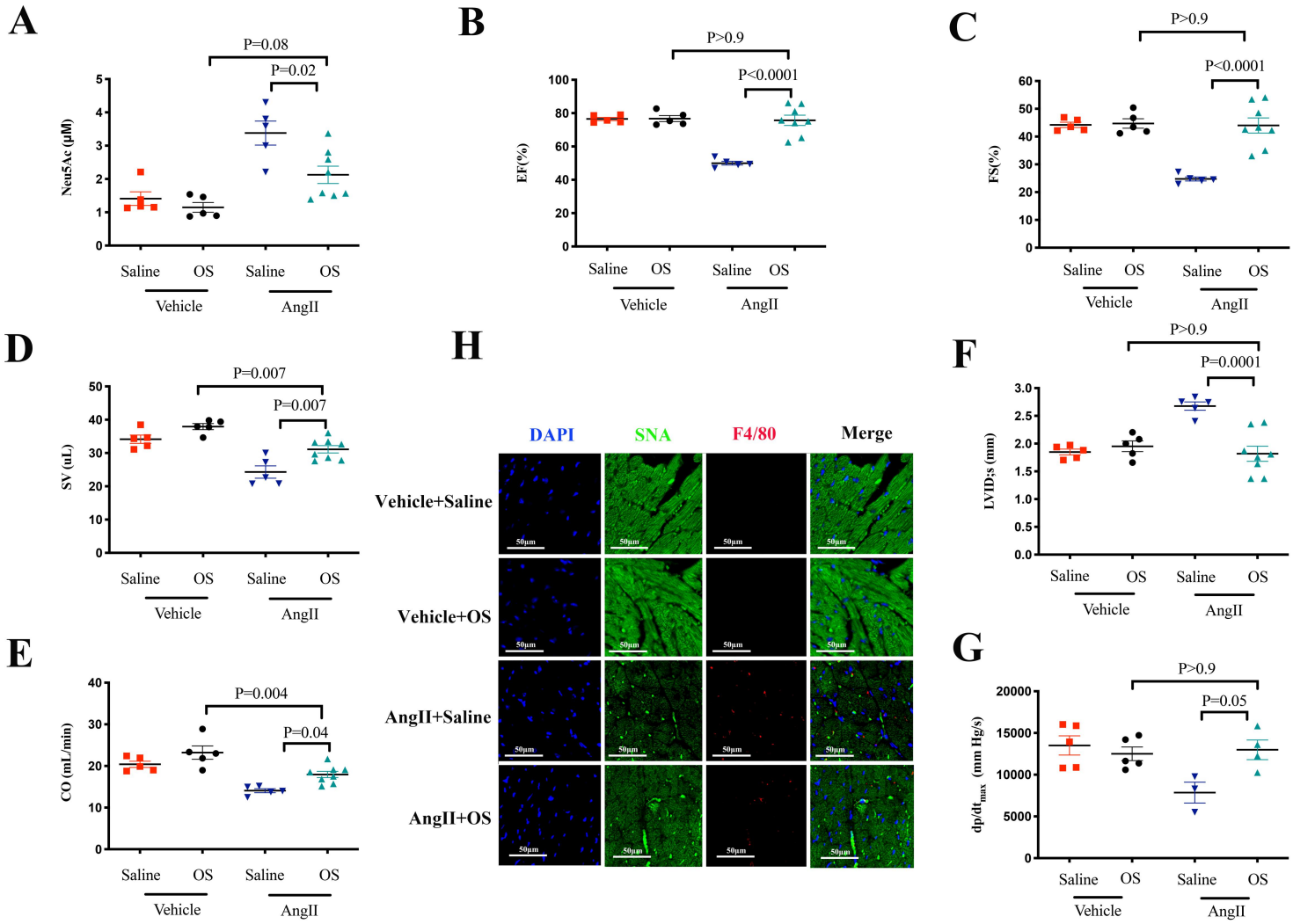

Figure VII. Oseltamivir phosphate inhibits desialylation and causes reversal of cardiac function in angiotensin II-induced HF mice. A, Free Neu5Ac concentration in the plasma detected by LC-MS (n=5-8 mice per group). The cardiac function at 4 weeks (**B** through **G**). **B**, Statistics of EF; **C**, Statistics of FS; **D**, Statistics of SV; **E**, Statistics of CO; **F**, Statistics of LVID;s by echocardiography (n=5-8 mice per group). **G**, Quantification of max dP/dt measured by aortic catheterization (n=3-5 mice per group). **H,** Representative histochemistry staining of α-(2,6)-linked sialic acid (SNA) (green) and F4/80 (ADGRE1) (red). Data are presented as mean ± SEM, and analyzed by One-way ANOVA with Bonferroni's test among four groups. Vehicle refers to the mice was infused with an equal volume of saline through subcutaneously implanted osmotic pumps as controls. Neu5Ac, N-acetylneuraminic acid; LC-MS, Liquid Chromatograph-Mass Spectrometry; OS, oseltamivir phosphate; EF, ejection fraction; FS, fractional shortening; SV, stroke

volume; CO, cardiac output; LVID;s, left ventricular internal diameter at systole; dP/dtmax, peak rate of pressure increase; Ang II, angiotensin II.

Supplemental Tables

Table I. Inclusion and exclusion criteria for heart failure patients.

Inclusion criteria	・NYHA functional Class ⩾II; ・Patients with reduced LVEF <40% (determined using transthoracic two-dimensional echocardiography); ・Patients are voluntary to be followed-up for more than 2 years and signed informed consent.
Exclusion criteria	・Significant valvar heart disease as the leading cause of HF; ・Severe liver dysfunction, renal dysfunction unrelated to HF; ・A history of cancer or pathological examination confirmed precancerous lesions that life expectancy < 1 year; ・Untreated second or third degree atrioventricular block; ・Acute myocardial infarction/unstable angina within the previous one month.

NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; HF, heart failure.

Table II. Inclusion and exclusion criteria for controls.

Inclusion criteria	・<50% diameter stenosis in any coronary artery confirmed by coronary angiogram; ・ Normal cardiac structure confirmed by transthoracic two-dimensional echocardiography; ・Without sever arrhythmia (such as ventricular tachycardia, second or third degree atrioventricular block, and sick sinus syndrome) confirmed by 12-lead ECG.
Exclusion criteria	・Refused to participate in the research work; ・ Liver and kidney dysfunction (total bilirubin is 1.5 times higher than the upper reference limit, AST or ALT is 2 times higher than the upper reference limit, serum creatinine ⩾ 2.0 mg/dl).

ECG, electrocardiogram.

Table III: The detailed parameters of targeted MS instrument and LC gradient.

Q TRAP5500	**Negative**
Spray voltage (kV)	4.5 ESI-
Source temperature (°C)	600
Collision activation parameter	Medium
Curtain Gas (psi)	20
GS1 (psi)	35
GS2 (psi)	50
LC Condition	
Column	Luna 5u Silica 100A, 2.0*150 mm
Column Chamber T (°C)	35°C
Flow Rate	0.5mL/min
Mobile Phase A	10mM NH4FA 1‰FA 100%H2O
Mobile Phase B	1‰FA 100%ACN
Gradient (A %)	0.01min-80% 1.00min-60% 3.50min-60% 3.51min-80% 7.00min-stop

Table IV: The internal standards were spiked in the samples and the accuracy of Neu5Ac concentration was calculated.

Standard concentrations(μM)	Accuracy (%)
0.078	94.2
0.156	98.7
0.312	100.2
0.625	101.4
1.25	102.4
2.5	101.2
5	99.6

Table V: Method reproducibility was confirmed by intra- and inter-day accuracy using samples at different concentrations of Neu5Ac.

Analyte	Spiked (µM)	Intra-day(n=6)		Inter-day(n=18)	
		Accuracy	RSD(%)	Accuracy	RSD(%)
Neu5Ac	0.5	94.8	4.58	92.3	5.15
	1	99.5	5.69	96.9	6.79
	5	95.5	2.43	95.4	5.73

RSD, relative standard deviation.

Table VI: Quality control samples with different Neu5Ac concentrations was measured every twenty samples. The calculated mean, CV% are given and the CV% values are below 10%.

	QC1	QC2	QC3	QC4	QC5
Mean(μM)	0.28	0.32	0.47	0.69	1.18
CV (%)	2.14	3.33	2.17	3.75	1.66

QC, Quality control; CV, coefficient of variation.

Table VII. Baseline demographic and clinical characteristics of the study controls.

	Overall (n=1700)
Demographics	
Age (years)	56 [50, 63]
Male, n (%)	771 (45)
History of diabetes mellitus, n (%)	130 (8)
History of hypertension, n (%)	532 (31)
History of stroke, n (%)	94 (6)
Smoking, n (%)	356 (21)
Drinking, n (%)	273 (16)
Clinical testing	
Systolic pressure (mmHg)	127 [115, 140]
Diastolic pressure (mmHg)	79 [71, 87]
Heart rate (beats/min)	76 [68, 83]
Creatinine (umol/L)	67 [57, 78]
ALT (U/L)	17 [13, 25]
AST (U/L)	19 [16, 24]
HDL cholesterol (mmol/L)	1.2 [1.0, 1.4]
LDL cholesterol (mmol/L)	2.3 [1.8, 2.9]

ALT, alanine transaminase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Continuous data are displayed as median (25% and 75% quartiles)**.**