

Soluble ST2 Links Inflammation to Outcome after Subarachnoid Hemorrhage

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Objective: To investigate whether soluble growth stimulation expressed gene 2 (sST2), a prognostic marker in cardiovascular and inflammatory disorders, is associated with neurological injury after aneurysmal subarachnoid hemorrhage (SAH).

Methods: We studied SAH patients from 2 independent cohorts. Outcome assessments included functional status at 90 days using the modified Rankin Scale (mRS), mortality, and delayed cerebral ischemia (DCI). The relationships between sST2 plasma level and outcome measures were assessed in both cross-sectional and longitudinal analysis. Primary blood mononuclear cells from SAH patients and elective aneurysm controls were analyzed by multiparameter flow cytometry.

Results: In the discovery cohort, sST2 predicted 90-day mRS 3–6 (C index = 0.724, $p < 0.001$) and mortality in Kaplan–Meier analysis ($p < 0.001$). The association with functional status was independent of age, sex, World Federation of Neurosurgical Societies score, modified Fisher score, treatment modality, and cardiac comorbidities (adjusted odds ratio = 2.28, 95% confidence interval = 1.04–5.00, $p = 0.039$). Higher sST2 concentration was observed in those patients with DCI (90.8 vs 53.7 ng/ml, $p = 0.003$). These associations were confirmed in a replication cohort. In patients with high sST2, flow cytometry identified decreased expression of CD14 ($4.27 \times 10^5 \pm 2,950$ arbitrary unit (AU) vs $5.64 \times 10^5 \pm 1,290$ AU, $p < 0.001$), and increased expression of CD16 ($39,960 \pm 272$ AU vs $34,869 \pm 183$ AU, $p < 0.001$).

Interpretation: Plasma sST2 predicts DCI, functional outcome, and mortality after SAH, independent of clinical and radiographic markers. Elevated sST2 is also associated with changes in CD14⁺CD16⁺ monocytes.

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Early and delayed brain injury after aneurysmal subarachnoid hemorrhage (SAH) contributes to a 30% mortality rate and leads to long-term disability in those who survive.^{1,2} There is a limited understanding of the molecular pathways involved in early brain injury,³ delayed cerebral ischemia (DCI),⁴ and long-term functional recovery.⁵ Although the calcium channel blocker nimodipine is approved for SAH,⁶ subsequent trials of cerebral vasodilators have not been shown to alter recovery. This paucity of treatment options highlights the need

to identify new biomarkers and candidate pathways that are linked to SAH-associated brain injury.

The immune response is thought to play a key role in the pathophysiology of SAH,⁷ and activation of proinflammatory pathways is involved in multiple sequelae including cerebral vasospasm and DCI.^{8–10} One candidate mechanism for regulation of immune response after injury is the growth stimulation expressed gene 2 (ST2)/interleukin (IL)-33 pathway. ST2 is a member of the IL-1 receptor family that is expressed on multiple immune cell types

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including T helper cells and macrophages.¹¹ IL-33 secreted by injured cells binds to transmembrane ST2 (tmST2), sequestering the signaling adaptor protein MyD88, preventing its interaction with toll-like receptor 4 (TLR4).¹² On microglia, monocyte-derived macrophages and helper T cells that express both tmST2 and TLR4,^{13,14} the binding of IL-33 to tmST2 attenuates the proinflammatory TLR4 cascade.¹² ST2 also exists as soluble form (sST2), which is generated by differential transcription through a dual promoter system.^{15,16} This alternate transcription has some degree of cell-type specificity, with hematopoietic cells predominantly expressing the transmembrane form and nonhematopoietic cells favoring the soluble form.¹⁷ sST2 acts as a decoy receptor, reducing the interaction of IL-33 with transmembrane ST2 and therefore shifting toward a proinflammatory response.^{11,18}

ST2 has been implicated in multiple immune-mediated diseases^{19–22} and also serves as a prognostic biomarker in myocardial infarction^{23,24} and heart failure.^{25,26} We recently identified sST2 as a marker of secondary brain injury and functional neurologic outcome after ischemic stroke,²⁷ suggesting it may also play a role in the immune response to neurovascular injury. In the current study, we evaluated sST2 as a marker of 90-day functional status and mortality in 2 independent SAH cohorts. To assess whether sST2 was linked to alterations in the immune response, we further explored the relationship between sST2 levels and immune cell subsets using multiparameter flow cytometry.

Patients and Methods

Patient Cohorts

Boston Cohort. Consecutive patients aged 18 years or older who presented with SAH between 2013 and 2016 were prospectively enrolled in the Massachusetts General Hospital (MGH) Acute Brain Injury Biobank. All patients or their surrogates provided informed consent following a protocol approved by the Partners Healthcare Institutional Review Board. Those with SAH due to trauma, mycotic aneurysm, or arteriovenous malformation (AVM) were excluded. A total of 190 patients with plasma samples available for analysis were identified. Clinical and demographic data were obtained through patient or surrogate interview and confirmed with medical records. SAH severity was classified using the World Federation of Neurological Societies (WFNS) grading scale,²⁸ and hemorrhage burden was classified using the modified Fisher score.²⁹ Plasma samples were available from early, intermediate, and late time points, corresponding to median postbleed time of 3, 8, and 13 days, respectively. In those patients who had external ventricular drains placed for clinical

indications, cerebrospinal fluid (CSF) was obtained at the same time points. Sonographic vasospasm was defined as peak systolic velocity > 200cm/s in the proximal anterior or middle cerebral arteries using transcranial Doppler sonography, per usual clinical practice. DCI was defined as a 2-point drop in Glasgow Coma Scale or a new focal deficit without another attributable cause for deterioration.³⁰ Functional status at 90 days was determined by telephone assessment of modified Rankin Scale (mRS) with patients and surrogates. Raters were not aware of sST2 levels at the time of assessment. Mortality data through 180 days were obtained from the medical record and confirmed using the Death Master File for the Social Security Administration.

Copenhagen Cohort. A total of 50 consecutive patients with aneurysmal SAH were prospectively enrolled at Rigshospitalet in Copenhagen, Denmark between November 2014 and May 2015. Patients or their surrogates provided informed consent for inclusion in this study, which was approved by the Danish Scientific Ethics Committee of the Capital Region. Patients were included who were ≥ 18 years of age, Danish speaking, and had clearly established symptom onset within 24 hours of presentation. Patients had daily plasma samples collected on postbleed days 1 through 9. Clinical and demographic data, including DCI designation, were collected in a manner analogous to that as described above for the MGH cohort.

Plasma and CSF sST2 Analysis

Blood was obtained at the time points described above, and within 1 hour of collection plasma was isolated and stored at –80°C for later analysis. When available, CSF was similarly collected and stored at –80°C. sST2 was measured in both sample types using a commercially available, Food and Drug Administration–approved enzyme-linked immunosorbent assay (ELISA; Presage ST2 Assay Kit; Critical Diagnostics, San Diego, CA). C-reactive protein (CRP) and IL-6 were also measured by ELISA (Thermo Fisher Scientific, Waltham, MA).

Flow Cytometry

Additional whole blood samples from a subset of patients from MGH were collected at the early time point for immune cell characterization. Blood was collected into BD Biosciences (San Jose, CA) Vacutainer CPT tubes, and peripheral blood mononuclear cells (PBMCs) were isolated. Samples were stained for the following markers: transmembrane ST2, CD45, CD3, CD4, CD8, CD45RA, CD19, CD56, CD16, CD14, CCR6, CCR4, PD-1, CCR7, CD127, CD28, CXCR3, CD25, HLA-

DR, and CD80. Samples were analyzed on a Beckman Coulter (Fullerton, CA) CytoFLEX LX Flow Cytometer at the MGH Pathology Flow Cytometry Core Facility.

Analysis of multiparameter flow cytometry was conducted using the *t*-distributed Stochastic Neighbor Embedding (tSNE) data reduction package in FlowJo 10 (FlowJo, Ashland, OR). Each sample was manually gated to identify singlets, and then further to select only viable cells based on staining with iFluor 860 maleimide (AAT Bioquest, Sunnyvale, CA). Cell populations were down sampled to 10,000 to 30,000 events per subject, and tSNE was performed using all parameters listed above. The resulting maps were visually compared and unique populations in each group identified. Changes in expression of specific markers was measured using mean fluorescence intensity. Differences in populations identified by particular marker expression were verified with manual gating.

Statistical Analysis

For baseline characteristics, continuous variables with a normal distribution are described as mean \pm standard deviation. Nonnormal or ordinal data are described as median and interquartile range (IQR). Differences in sST2 between groups at a single time point were determined using Wilcoxon rank sum or Kruskal-Wallis tests as appropriate, with Dunn's test performed for post hoc multiple comparisons. In the MGH cohort, differences in sST2 between groups over time were determined using linear mixed-effects models with a random effect for subject. sST2 concentration was log-transformed prior to

analysis. In the Copenhagen cohort, which used a balanced design of regular daily sample collection, differences between groups over time were compared using an analysis of response profiles.³¹ Cross-sectional analysis in the Copenhagen cohort was conducted using day 3 samples, which was prespecified to correspond to the early time point in the Boston cohort. The predictive value of sST2 on mRS was determined using receiver operating characteristic (ROC) curve analysis with ideal cut points identified using Youden index. Multivariate models of mRS and mortality prediction were calculated using log-transformed sST2 values alongside other clinical variables in logistic regression models. Association of sST2 with mortality was determined using Kaplan–Meier log-rank analysis. All analyses were carried out in Stata version 15 (StataCorp, College Station, TX).

Results

Patient Characteristics

Characteristics of the Boston and Copenhagen cohorts are presented in Table 1. The 190 patients in the Boston cohort had a mean age of 57 ± 11 years, median WFNS grade of 1 (IQR = 1–4) and modified Fisher grade of 3 (IQR = 3–4). Compared to the Boston patients, the Copenhagen cohort was more predominantly female (90 vs 62% in Boston) and had a higher WFNS grade (median = 3 [IQR = 1–4]; $p = 0.016$), but outcomes were better with a median 90-day mRS of 1 (IQR = 0–3) in Copenhagen versus 2 (IQR = 1–3) in Boston ($p = 0.045$). Plasma sST2

TABLE 1. Characteristics of the Boston and Copenhagen Cohorts

	Boston, n = 190	Copenhagen, n = 50	<i>p</i>
Age, yr	57 \pm 12	61 \pm 11	0.07
Sex, female	117 (62%)	45 (90%)	<0.001
WFNS [IQR]	1 [1–4]	3 [1–4]	0.016
Modified Fisher score [IQR]	3 [3, 4]	3 [3–3]	0.25
mRS at 90 days [IQR]	2 [1, 3]	1 [0–3]	0.045
Mortality at 90 days	25 (13%)	5 (10%)	0.55
DCI ^a	61 (43%)	25 (50%)	0.39
Plasma sST2, ng/ml, day 3 [IQR]	75.1 [44.0–133]	82.4 [47.8–167]	0.73
CSF sST2, ng/ml, day 3 [IQR] ^b	17.1 [4.1–44.1]	—	—

^aDCI designations were available for 142 patients in the MGH cohort.

^bCSF samples were available from 46 patients in the MGH cohort.

CSF = cerebrospinal fluid; DCI = delayed cerebral ischemia; IQR = interquartile range; MGH = Massachusetts General Hospital; mRS = modified Rankin Scale; sST2 = soluble ST2; WFNS = World Federation of Neurosurgical Societies.

measured at the early time point (median = 3 days after ictus) in Boston was similar to day 3 sST2 in Copenhagen (75.1ng/ml vs 82.4ng/ml, $p = 0.73$).

Plasma sST2 Is Elevated in SAH and Is Associated with Outcome

Plasma sST2 levels were measured in healthy volunteers ($n = 12$), patients undergoing elective clipping of unruptured aneurysms ($n = 13$), those with arteriovenous malformation ($n = 6$), and those with spontaneous SAH.

Spontaneous SAH patients were further subdivided into those with a perimesencephalic pattern ($n = 18$) and a more diffuse, classical aneurysmal SAH pattern ($n = 93$). Patients with aneurysmal SAH had significantly higher levels of sST2 measured a median of 3 days after ictus compared to those with AVM, perimesencephalic blood, following elective clipping, or in healthy controls (median = 80.1ng/ml [IQR = 45.7–139.4] vs 17.8 [IQR = 9.24–50.1], 35.3 [IQR = 27.6–40.2], 24.9 [IQR = 16.6–30.0], and 25.6 [IQR = 18.8–32.1],

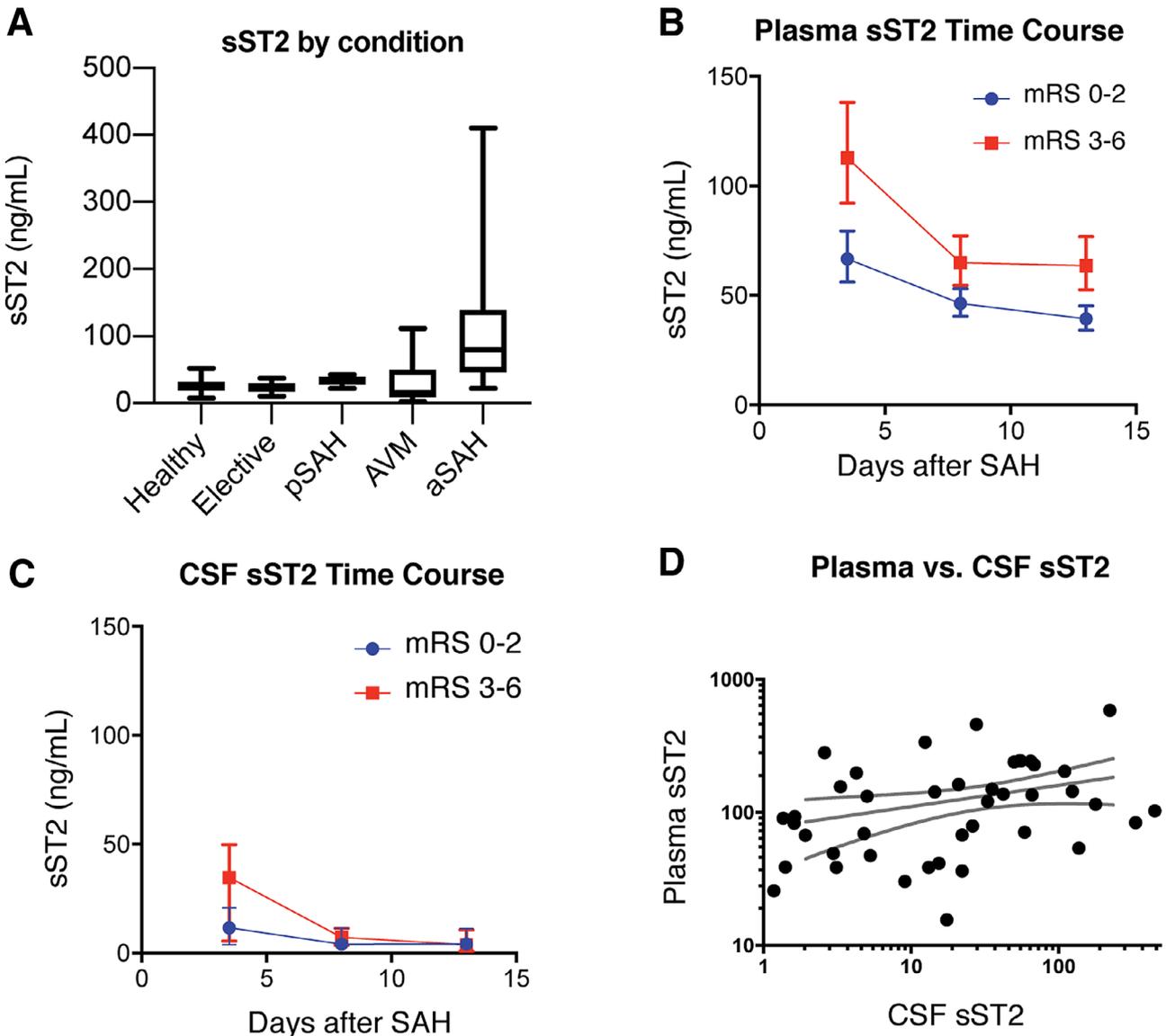
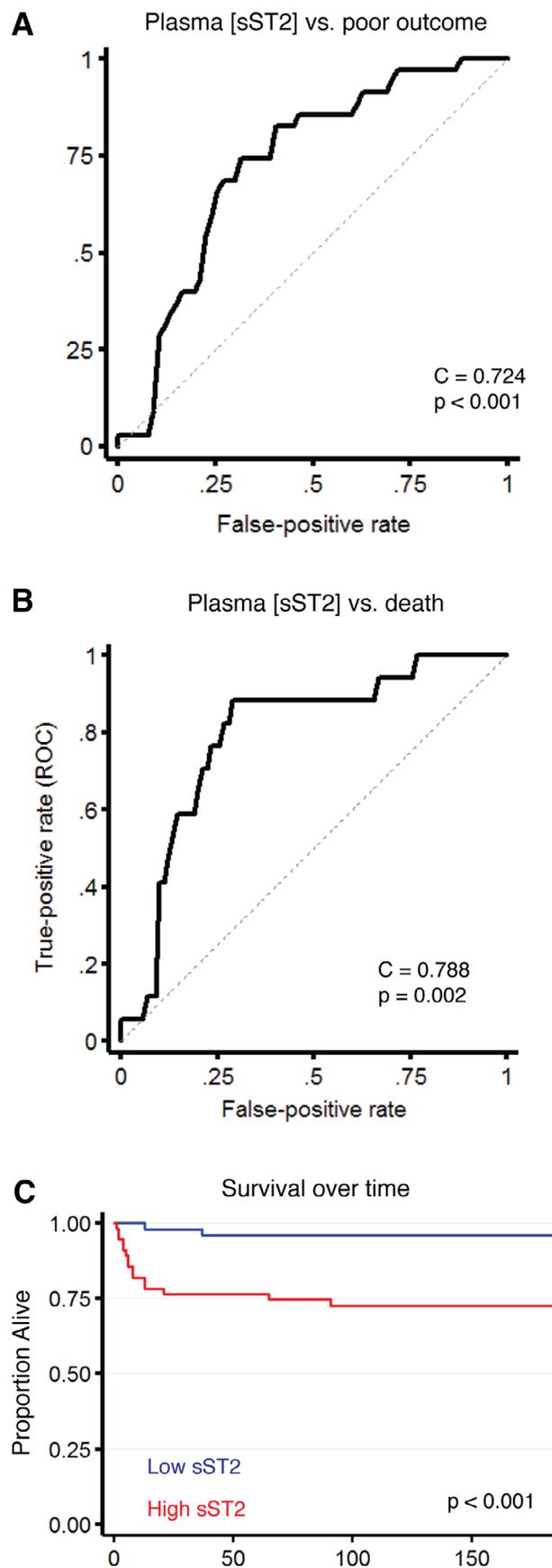


FIGURE 1: Plasma and CSF sST2 in the Boston Cohort. (A) Plasma sST2 is elevated in those patients with aneurysmal SAH compared to those with arteriovenous malformation, perimesencephalic pattern SAH, patients undergoing elective clipping of an unruptured aneurysm, or healthy volunteers ($p < 0.0001$). (B) Plasma sST2 is associated with outcome, with significantly higher sST2 in those with poor (mRS 3–6, red squares) compared to good outcome (mRS 0–2, blue circles) using a linear mixed-effects model ($\beta = 0.54$, 95% CI = 0.25–0.83, $p < 0.001$). Markers indicate mean with error bars showing standard deviation. (C) A similar pattern was seen in CSF sST2 levels, but there was no significant difference based on outcome ($\beta = 0.61$, 95% CI = –0.43 to 1.64, $p = 0.25$). (D) Plasma and CSF sST2 are positively correlated (Spearman $r = 0.37$, $p = 0.012$). CI = confidence interval; CSF = cerebrospinal fluid; mRS = modified Rankin Scale; SAH = subarachnoid hemorrhage; sST2 = soluble ST2. [Color figure can be viewed at www.annalsofneurology.org]



respectively; adjusted $p < 0.0001$ for aneurysmal SAH vs all other conditions; Fig 1A).

In the Boston cohort, 305 plasma samples were collected from the 190 patients across all 3 time points. Plasma sST2 levels were highest at the early time point and decreased at the middle and late time points (see Fig 1B). Those patients with poor (mRS 3–6) outcome had significantly higher sST2 as compared to those with good (mRS 0–2) outcome ($\beta = 0.54$, 95% confidence interval [CI] = 0.25–0.83, $p < 0.001$). A total of 144 CSF samples were obtained from 46 patients in the Boston cohort. A similar pattern was observed for CSF sST2 levels, but there was no significant difference based on outcome (see Fig 1C; $\beta = 0.61$, 95% CI = –0.43 to 1.64, $p = 0.25$). However, plasma and CSF sST2 concentrations at the early time point were moderately correlated (Spearman $r = 0.37$, $p = 0.012$; see Fig 1D).

Early Plasma sST2 Is an Independent Predictor of Outcome and Survival in SAH Patients

To further explore the association between sST2 and clinical outcome, we compared early time point sST2 concentration to functional neurologic status and mortality. Elevated plasma sST2 was associated with poor outcome (median = 112.6ng/ml [IQR = 74.0–176.4] for mRS 3–6 vs 55.5ng/ml [IQR = 39.1–100.4] for mRS 0–2, $p = 0.0002$). Similar elevations were seen in plasma IL-6 (median = 29.6pg/ml [IQR = 13.7–51.4] for mRS 3–6 vs 14.2pg/ml [IQR = 6.4–30.0] for mRS 0–2, $p = 0.011$) and CRP (29.5 μ g/ml [IQR = 9.38–95.9] for mRS 3–6 vs 2.24 μ g/ml [IQR = 1.40–6.60] for mRS 0–2, $p = 0.021$). sST2 remained an independent predictor of poor outcome in multivariate logistic regression models including either IL-6 (adjusted odds ratio [OR] for sST2 is 2.06 [IQR = 1.03–4.15], $p = 0.042$; adjusted OR for IL-6 is 1.47 [IQR = 0.92–2.33], $p = 0.103$) or CRP (adjusted OR

FIGURE 2: Plasma sST2 is associated with outcome and mortality in the Boston cohort. (A) ROC analysis finds early sST2 to be a predictor of poor outcome (mRS 3–6) at 90 days (C statistic = 0.724, 95% CI = 0.620–0.827, Youden cut point = 80.8ng/ml, $p < 0.001$). (B) Similar results are observed for mortality, where early sST2 is again a predictor of outcome (C statistic = 0.788, 95% CI = 0.681–0.896, Youden cut point = 91.3ng/ml, $p = 0.002$). (C) Kaplan-Meier analysis of patients with high versus low sST2 (dichotomized based on the median level at the first time point) found those with high sST2 to have a significantly higher rate of death (D; $p < 0.001$). CI = confidence interval; mRS = modified Rankin Scale; ROC = receiver operating characteristic; sST2 = soluble ST2. [Color figure can be viewed at www.annalsofneurology.org]

TABLE 2. Univariate and Multivariate Models for Prediction of Functional Outcome and Mortality

	Poor Outcome, mRS 4–6			Mortality		
	sST2 OR	95% CI	<i>p</i>	sST2 OR	95% CI	<i>p</i>
Model 1	3.02	1.58–5.76	<0.001	4.62	1.92–11.1	<0.001
Model 2	2.31	1.13–4.72	0.022	4.26	1.43–12.7	0.009
Model 3	2.29	1.04–5.01	0.039	3.54	1.13–11.1	0.03
Model 4	2.28	1.05–5.00	0.039	3.19	1.00–10.2	0.05

Model 1: sST2 univariate. Model 2: Model 1 + age, sex, WFNS. Model 3: Model 2 + modified Fisher, perimesencephalic appearance, clip versus coil. Model 4: Model 3 + atrial fibrillation, congestive heart failure.

CI = confidence interval; mRS = modified Rankin Scale; OR = odds ratio; sST2 = soluble ST2.

for sST2 is 2.49 [IQR = 1.28–4.85], $p = 0.007$; adjusted OR for CRP is 1.37 [IQR = 1.03–1.81], $p = 0.029$).

ROC analysis found sST2 to be a significant predictor of poor outcome, with an optimal sST2 cut point of 80.8ng/ml (Fig 2A; C index = 0.724, 95% CI = 0.620–0.827, $p < 0.001$). Plasma sST2 was similarly predictive of mortality (see Fig 2B; C index = 0.788, 95% CI = 0.681–0.896, Youden cut point: 91.3ng/ml, $p = 0.002$). Kaplan–Meier analysis of patients with high versus low sST2 (dichotomized based on the median level at the first time point) found those with high sST2 to have a significantly higher risk of death (see Fig 2C; $p < 0.001$).

To determine the independence of sST2 as a predictor of functional status and mortality, we constructed multivariate logistic regression models (Table 2). In univariate analysis, log-transformed sST2 at the early time point was associated with poor outcome (OR = 3.02, 95% CI = 1.58–5.76, $p < 0.001$) and mortality (OR = 4.62, 95% CI = 1.92–11.1, $p < 0.001$). sST2 remained an independent predictor of mRS and mortality after adjusting for age, sex, and WFNS score (mRS 3–6: adjusted OR = 2.31 [IQR = 1.13–4.72], $p = 0.022$; mortality: adjusted OR = 4.26 [IQR = 1.43–12.7], $p = 0.009$). When including radiographic markers (modified Fisher score and perimesencephalic pattern) and treatment type (aneurysm clipping vs coiling), sST2 remained an independent predictor (poor outcome: adjusted OR = 2.29, 95% CI = 1.04–5.01, $p = 0.039$; mortality: adjusted OR = 3.54, 95% CI = 1.13–11.1, $p = 0.03$). Given the known role of sST2 as a cardiac biomarker,^{23,32} we further adjusted for a history of atrial fibrillation or congestive heart failure, and sST2 again remained an independent predictor of outcome (adjusted OR = 2.28, 95% CI = 1.04–5.00, $p = 0.039$) and mortality (adjusted OR = 3.19, 95% CI = 1.00–10.2, $p = 0.05$).

sST2 Levels Are Associated with Outcome in a Replication Cohort

To replicate these findings in an independent SAH cohort, we measured sST2 in plasma samples from SAH

patients enrolled in an observational study from Rigshospitalet, Copenhagen, Denmark. Samples were collected daily, up to postbleed day 9, for a total of 374 samples from the 50 patients. Similar to the discovery cohort (as shown in Fig 1B), patients with poor outcome had a higher sST2 over time (Fig 3A; $p = 0.002$). sST2 was similarly higher over time in those who died (see Fig 3B; $p < 0.0001$). To parallel our analysis of sST2 at the early time point versus outcome in the Boston cohort (see Fig 2A), we compared sST2 at day 3 between those with good and poor outcome in the Copenhagen cohort, and again found higher sST2 concentration in those with poor 90-day mRS (see Fig 3C; $p = 0.012$).

Early sST2 Level Predicts Delayed Cerebral Ischemia

To explore the link between sST2 and secondary neurologic injury, we examined the relationship between early time point sST2 and sonographic vasospasm, and between sST2 and DCI in the Boston cohort. Although the presence of sonographic vasospasm was associated with risk for DCI in the Boston cohort (risk ratio = 2.4, 95% CI = 1.26–4.43, $p = 0.003$), there was no difference in early time point sST2 between those with and without vasospasm (79.0 vs 74.4ng/ml, $p = 0.476$; Fig 4A). There was, however, significantly higher early sST2 in those with DCI (90.8 vs 53.7ng/ml, $p = 0.003$; see Fig 4B). Similar results were seen in the Copenhagen cohort, where sST2 over time was higher in those with DCI (see Fig 4C; $p = 0.049$). Elevated sST2 at the early time point predicted DCI in the MGH cohort with an optimal cut point of 76.7ng/ml by Youden index (C index = 0.668, 95% CI = 0.548–0.789, $p = 0.034$).

High sST2 Is Associated with Changes in Immune Cell Populations

We next sought to explore the differences in immune cell populations associated with SAH. We first compared

PBMCs from aneurysmal SAH and control patients at the time of elective aneurysm clipping (n = 6 for each group, 30,000 cells per patient). The tSNE maps (Fig 5A) identified a cell population selectively increased in aneurysmal SAH that expressed transmembrane ST2, CD14, and in subpopulations, CD16 or CD56. Manual gating found the proportion of cells expressing transmembrane ST2 and

CD16 to be increased in SAH patients compared to elective clipping cases (38.0 ± 6.7% in SAH [n = 6] vs 23.0 ± 3.15% in controls [n = 13], p = 0.035). There were also higher proportions of ST2⁺CD14⁺ (37.7 ± 14.7% vs 17.0 ± 3.5%, p = 0.095) and ST2⁺CD56⁺ populations (25.6 ± 3.3% vs 22.1 ± 3.1%, p = 0.494), but these differences were not significant.

To further examine whether these changes were also related to sST2 expression, the aneurysmal rupture SAH patients were divided into low- and high-sST2 groups, and PBMCs from patients in each group were again compared using tSNE. The resulting maps (see Fig 5B) highlighted a shift in expression of transmembrane ST2, CD14, and CD16 (circled in Fig 5B left-most panels). This was confirmed by comparison of mean fluorescence intensity (see Fig 5C), where high sST2 was associated with lower tmST2 (43,027 ± 860 vs 86,500 ± 368, p < 0.001), lower CD14 (4.27 × 10⁵ ± 2,950 vs 5.64 × 10⁵ ± 1,290, p < 0.001), and higher CD16 (39,960 ± 272 vs 34,869 ± 183, p < 0.001). No changes were seen in expression of T cell markers, which has been associated with sST2 in other conditions,^{33,34} a finding that was confirmed by manual gating for type 1 helper T cells (Th1) (16.7 ± 0.75% in low sST2 vs 17.1 ± 2.1% in high sST2, p = 0.845) and Th2 (7.0 ± 0.46% in low vs 5.8 ± 0.69% in high, p = 0.191) subsets. In addition, tSNE identifies 2 populations of CD56⁺ cells that were differentially present in low- and high-sST2 patients (circled in rightmost panels of Fig 5). The population present in low sST2 was CD56⁺CD14⁺ but CD16⁻, whereas that present in high sST2 was CD56⁺CD16⁺ but CD14⁻.

Potential changes in CD56⁺ natural killer (NK) cell subsets were verified by manually gating the entire PBMC population in each aneurysmal SAH sample to identify the proportion of PBMCs expressing each subgroup of markers (see Fig 5D). High sST2 was associated with a higher proportion of CD16⁺CD56⁺ cells, although this difference was not

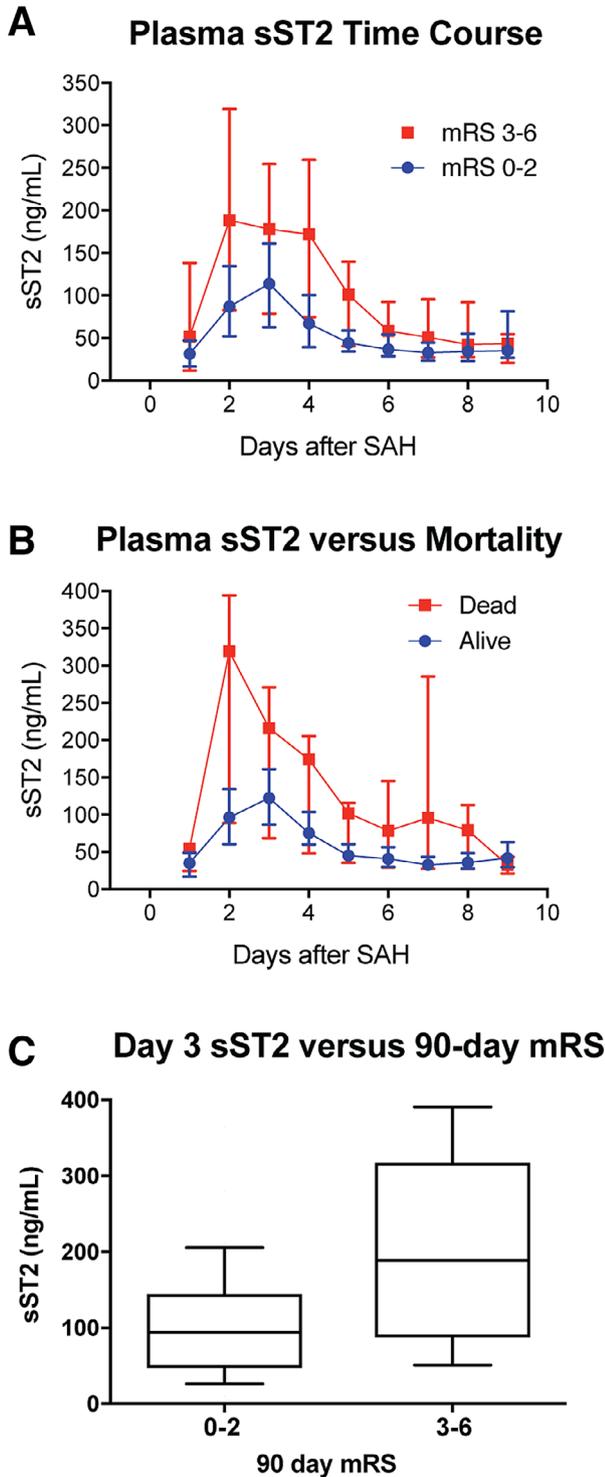
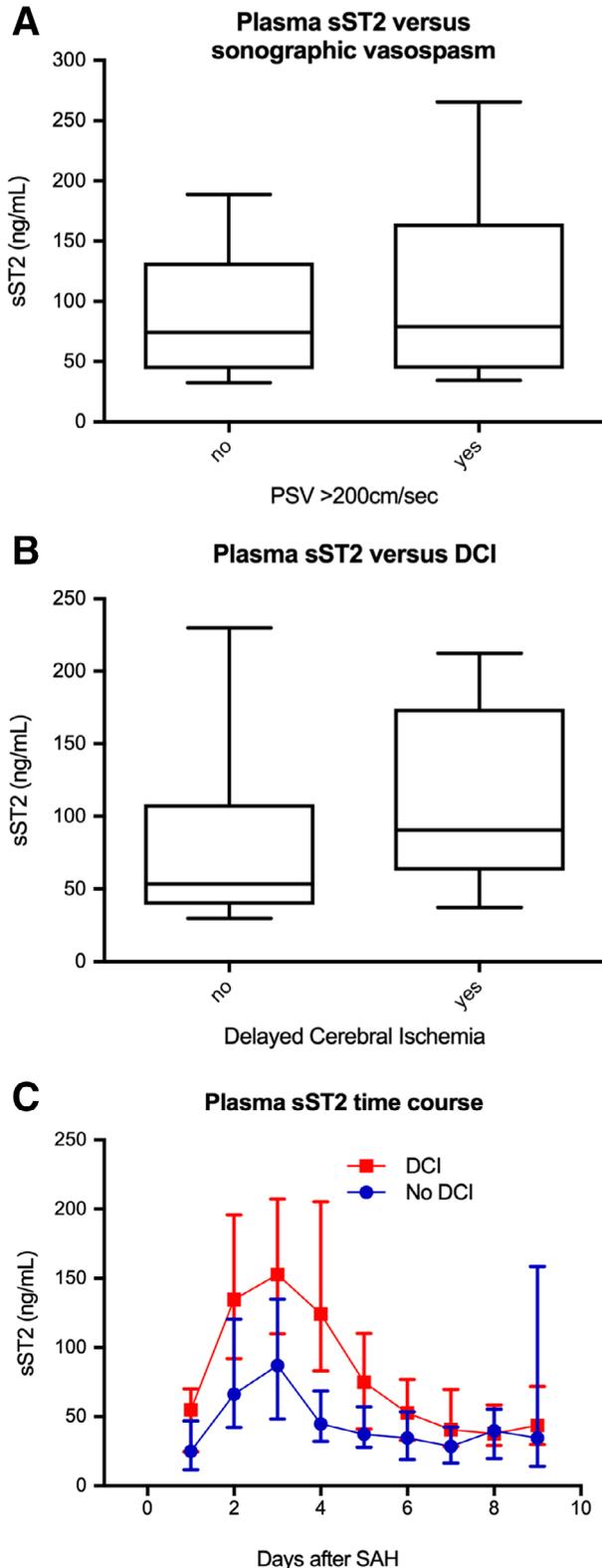


FIGURE 3: Plasma sST2 and outcome in the Copenhagen cohort. (A) sST2 over time was also significantly higher in those with poor outcome in the Copenhagen cohort (analysis of response profiles, p = 0.002). (B) Similar results were seen for mortality, with sST2 again higher over time in those who died, but this difference did not meet the threshold for significance (p < 0.0001). (C) In an effort to replicate the Boston cohort results, sST2 concentration at day 3 after ictus, corresponding to the median day of the early time point in the Boston cohort, was compared between those with good and poor outcome. Median day 3 sST2 concentration was higher in those with poor outcome (188.5ng/ml [IQR = 87.4–317.6] vs 94.3 ng/ml [IQR = 47.2–114.5], p = 0.012). IQR = interquartile range; sST2 = soluble ST2. [Color figure can be viewed at www.annalsofneurology.org]

significant ($8.2 \pm 4.21\%$ vs $18.1 \pm 2.4\%$, $p = 0.11$). There was a significantly smaller proportion of $CD14^+CD56^+$ cells in those patients with high sST2 ($19.8 \pm 0.33\%$ in low sST2 vs $2.4 \pm 1.3\%$ in high ST2, $p = 0.0002$).



Discussion

In this study, we demonstrate that the plasma level of sST2 is predictive of functional outcome and mortality after subarachnoid hemorrhage. The predictive ability of sST2 was independent of clinical and radiographic factors including age, WFNS, modified Fisher scale, cardiac comorbidities, and treatment modality. These findings were replicated in a second independent cohort of SAH patients. These associations were present in longitudinal as well as cross-sectional analysis early in the hospital course. This latter point demonstrates the potential of sST2 to serve as a clinically relevant biomarker with early levels predicting subsequent clinical events. This is highlighted by the association between day 3 sST2 and subsequent development of DCI. Interestingly, sST2 was not associated with sonographic vasospasm, adding further support to the concept that sonographic changes are poorly correlated with clinical findings,^{35,36} and that other pathophysiologic mechanisms may contribute to clinical deterioration after SAH.

One proposed contributor to delayed deterioration and poor outcome after SAH is activation of the immune response.³⁷⁻⁴¹ Consistent with this, we found that aneurysm rupture and high levels of sST2 after rupture were associated with shifts in immune cell populations. Our comparison between SAH patients and control patients undergoing elective aneurysm clipping found an apparent shift in a $CD14^+$ monocyte population, which is consistent with animal studies of SAH.^{42,43} Human studies have similarly shown increases in monocyte-associated genes after aneurysm rupture,⁴⁴ elevations in monocyte chemoattractant protein 1 in serum⁴⁵ and CSF⁴⁶ after SAH, and increase in activated monocytes identified by CSF and peripheral blood flow cytometry.⁷ We further found that in those SAH patients with high sST2, there

FIGURE 4: sST2 levels are associated with DCI. (A) No association was seen between presence of sonographic vasospasm (defined as peak middle cerebral artery velocity > 200cm/s) and median plasma sST2 at the early time point (79.0 vs 74.4ng/ml, $p = 0.476$). (B) There was, however, higher day 3 sST2 in those patients with DCI, defined as a clinical worsening of at least 2 points on the GCS scale over 24 hours (90.8 vs 53.7ng/ml, $p = 0.003$). (C) The relationship between sST2 and DCI was also present in the Copenhagen cohort, where those with DCI had significantly higher sST2 concentration over time (analysis of response profiles, $p = 0.049$). Markers indicate mean sST2, error bars show standard deviation. (D) Elevated early time point sST2 predicted DCI in the Boston cohort with an optimal cut point of 76.7ng/ml by Youden index (C index = 0.668, 95% CI = 0.548–0.789, $p = 0.034$). DCI = delayed cerebral ischemia; GCS = Glasgow Coma Scale; sST2 = soluble ST2. [Color figure can be viewed at www.annalsofneurology.org]

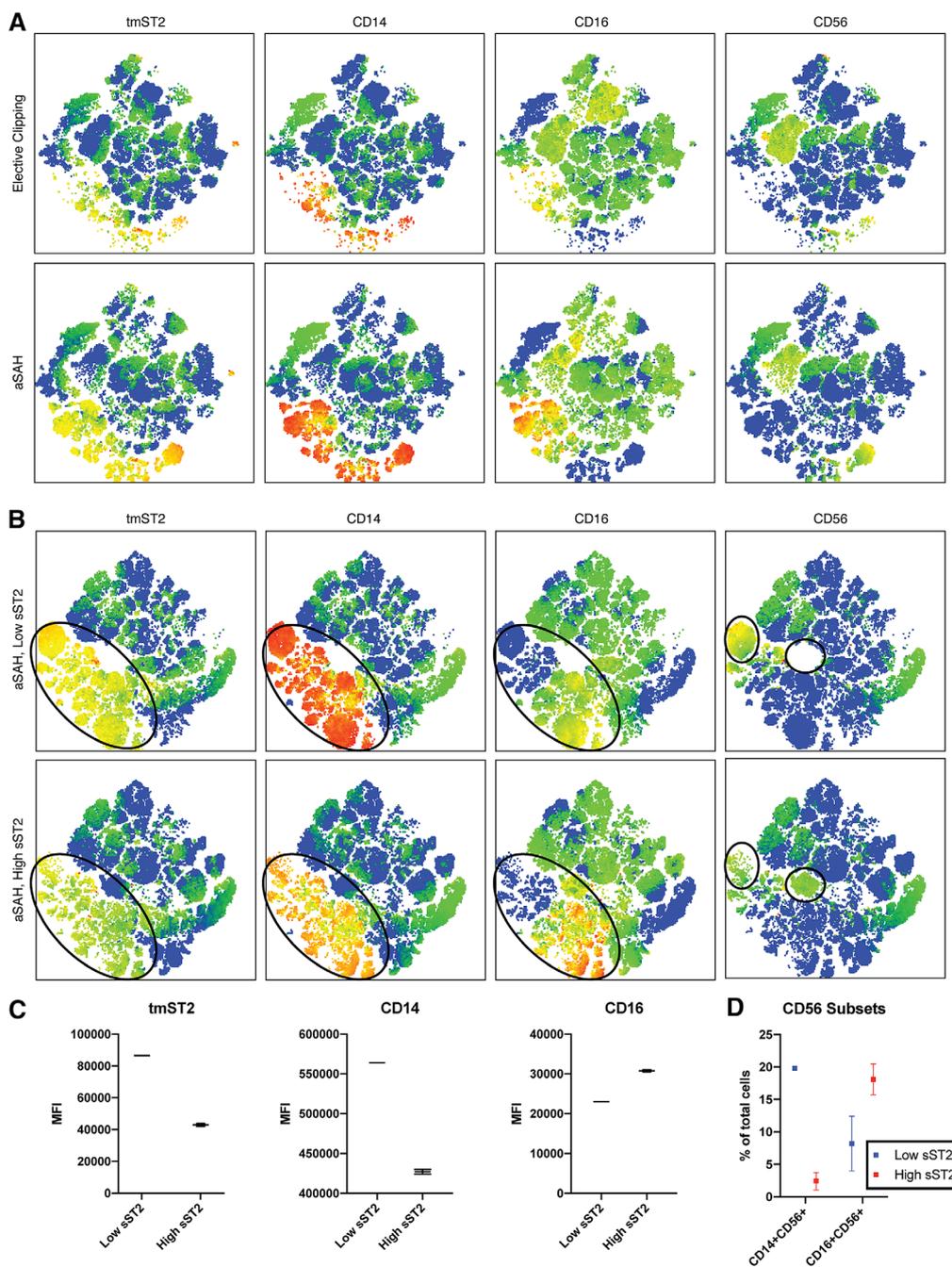


FIGURE 5: Flow cytometric analysis of subarachnoid hemorrhage patients. (A) t-distribution Stochastic Neighbor Embedding (tSNE) analysis of subjects with aneurysmal SAH ($n = 6$) and control patients undergoing elective clipping ($n = 6$). Patients with aneurysmal rupture appear to have an increase in cells expressing transmembrane ST2 (tmST2) as well as the monocyte marker CD14, with subpopulations of CD16 and CD56 positive cells. **(B)** tSNE analysis of aneurysmal rupture subjects with low sST2 versus high sST2 at the early time point (defined as below or above the median value of 75ng/ml, $n = 3$ in each group). There is a shift in expression of tmST2 (circled population in 3 left panels), with lesser intensity in the high ST2 group. This population also appears to have lower CD14 and variably increased CD16 expression. Unique cell clusters expressing NK cell marker CD56 were identified in low-sST2 and high-sST2 populations (circled clusters in right-most panels) variably expressing CD14 and CD16. **(C)** Analysis of mean fluorescence intensity within the subpopulation of cells identified by tSNE analysis in the aneurysmal rupture patients finds that high sST2 is associated with lower tmST2 ($43,027 \pm 860$ vs $86,500 \pm 368$, $p < 0.001$), lower CD14 ($4.27 \times 10^5 \pm 2950$ vs $5.64 \times 10^5 \pm 1290$, $p < 0.001$), and higher CD16 expression ($39,960 \pm 272$ vs $34,869 \pm 183$, $p < 0.001$). Markers indicate mean; error bars show standard error. **(D)** Manual gating of CD56⁺ cell subpopulations in aneurysmal rupture patients finds lower percentage of CD14⁺CD56⁺ ($19.85 \pm 0.33\%$ in low sST2 vs $2.4 \pm 1.3\%$ in high ST2, $p = 0.0002$) and higher percentage of CD16⁺CD56⁺ cells in the high-sST2 patients ($8.2 \pm 4.21\%$ vs $18.1 \pm 2.4\%$, $p = 0.11$). NK = natural killer; SAH = subarachnoid hemorrhage; sST2 = soluble ST2.

was a reduction in CD14 expression intensity and increase in CD16 expression as well as a shift in a CD56⁺ cell population from CD56⁺CD14⁺ to CD56⁺CD16⁺. CD56⁺ is a marker of NK cells, and CD16⁺ is an activating receptor on those cells.⁴⁷ CD14⁺CD56⁺ cells, which also express HLA-DR (data not shown), are thought to be a precursor of dendritic cells, and thus are involved in initiation of the adaptive immune response.^{48,49} Increased peripheral blood NK cells have been reported in patients undergoing clipping for ruptured aneurysm, particularly in those with poor outcome.⁵⁰ NK cell subset analysis has similarly shown increased CD16⁺ NK cell populations in the CSF of SAH patients with vasospasm.⁵¹ Although sST2 has been associated with shifts in T helper cell subsets in autoimmune diseases,^{11,19,22} we did not observe any association between sST2 level and T cell populations in this study. Taken together, our data support alterations in the innate immune response, including monocytes and NK cells, in relation to SAH and increased sST2 level.

This study has several limitations. It was a retrospective analysis, although we used 2 independent, prospectively collected cohorts that allowed for replication. Both cohorts consisted of consecutively enrolled patients, but not all patients consented, which has potential to introduce bias. Moreover, the 2 cohorts did differ, where the Copenhagen cohort was predominantly female, and the Boston cohort was more evenly distributed. The Copenhagen cohort had a higher initial WFNS grade, but better median 90-day outcome. Despite these differences, we found similar relationships between sST2, DCI, and outcome in the two cohorts. Our flow cytometry analysis was based on a panel design that surveyed a broad array of markers to phenotype as many immune cell types as possible. Although this has the advantage of broadly phenotyping immune cells, it limited our ability to further specify the monocyte subtypes that were altered with high sST2. Future study examining multiple time points and using additional monocyte/macrophage and NK cell markers with techniques such as mass cytometry will be needed to further characterize these changes.

In summary, we find that plasma sST2 measured early after aneurysm rupture is able to predict DCI, functional neurologic outcome, and mortality. Furthermore, elevated sST2 is associated with shifts toward a more proinflammatory population of monocytes and NK cells in patients with SAH. These findings have important implications not only for guiding risk stratification of patients with SAH, but also provide a pathway for further investigation into mechanisms of neurological deterioration after aneurysm rupture that may inform the design of novel therapeutic targets.

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Author Contributions

M.B.B., Z.W., S.B., C.H., C.S., R.M., M.J.K., A.B.P., and K.M. contributed to acquisition and analysis of data. M.B.B., Z.W., and W.T.K. contributed to drafting of the manuscript and preparation of the figures. W.T.K. contributed to the conception and design of the study.

Potential Conflicts of Interest

Nothing to report.

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