REGULAR ARTICLE

blood advances

A biomarker panel for risk of early respiratory failure following hematopoietic cell transplantation

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Key Points

- This study identified and validated ST2, WFDC2, IL-6, and TNFR1 as risk biomarkers for RF and related mortality post-HCT.

Plasma biomarkers associated with respiratory failure (RF) following hematopoietic cell transplantation (HCT) have not been identified. Therefore, we aimed to validate early (7 and 14 days post-HCT) risk biomarkers for RF. Using tandem mass spectrometry, we compared plasma obtained at day 14 post-HCT from 15 patients with RF and 15 patients without RF. Six candidate proteins, from this discovery cohort or identified in the literature, were measured by enzyme-linked immunosorbent assay in day-7 and day-14 post-HCT samples from the training (n = 213) and validation (n = 119) cohorts. Cox proportional-hazard analyses with biomarkers dichotomized by Youden's index, as well as landmark analyses to determine the association between biomarkers and RF, were performed. Of the 6 markers, Stimulation-2 (ST2), WAP 4-disulfide core domain protein 2 (WFDC2), interleukin-6 (IL-6), and tumor necrosis factor receptor 1 (TNFR1), measured at day 14 post-HCT, had the most significant association with an increased risk for RF in the training cohort (ST2: hazard ratio [HR], 4.5, $P = .004$; WFDC2: HR, 4.2, $P = .010$; IL-6: HR, 6.9, $P < .001$; and TFNR1: HR, 6.1, $P < .001$) and in the validation cohort (ST2: HR, 23.2, $P = .013$; WFDC2: HR, 18.2, $P = .019$; IL-6: HR, 12.2, $P = .014$; and TFNR1: HR, 16.1, $P = .001$) after adjusting for the conditioning regimen. Using cause-specific landmark analyses, including days 7 and 14, high plasma levels of ST2, WFDC2, IL-6, and TNFR1 were associated with an increased HR for RF in the training and validation cohorts. These biomarkers were also predictive of mortality from RF. ST2, WFDC2, IL-6 and TNFR1 levels measured early posttransplantation improve risk stratification for RF and its related mortality.

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The original mass spectrometry data files, the Proteome Discoverer output files, and protein database FASTA files are available for download from MassIVE (http://massive.ucsd.edu) using identifier MSV000087586. Requests for other data should be sent to Courtney M. Rowan (coujohns@iu.edu).

The full-text version of this article contains a data supplement.

Introduction

Allogeneic hematopoietic cell transplantation (HCT) is a life-saving therapy used for malignant and nonmalignant diseases. However, post-HCT pulmonary complications continue to be a significant problem.[1-4] When severe, pulmonary complications can result in respiratory failure (RF), affecting 10% to 23% of patients.[5-7] Acute RF, requiring mechanical ventilation, carries a very high mortality rate in this population.[4,8,9] Although survival has improved, the risk of death with mechanical ventilation remains unacceptably high, leaving physicians who care for these patients with appropriate concerns regarding the timing of intensive care unit (ICU) transfer and intubation.[10] Further complicating treatment is the fact that the cause of RF is unknown in up to 15% of cases, making the institution of standard therapies challenging.[11-13] The ability to determine who is at highest risk for RF may allow for earlier supportive care intervention, prompt involvement of a critical care team, intensive monitoring, improvement in family/patient counseling, and guidance with regard to the appropriate use of invasive supportive strategies, subsequently improving survival.[8,14] The significance of RF occurring after HCT was recently underscored by a 2018 National Institutes of Health workshop that was specifically convened to identify clinical challenges and gaps in the scientific knowledge with regard to pulmonary dysfunction after HCT in children.[15]

Although clinical models, such as the Pediatric Early Warning Score, have demonstrated an association with the need for critical care in this population,[16-19] they are not designed for RF and are often assessed/implemented too late in the medical course. The Biomarkers, Endpoints, and other Tools resource from the US Food and Drug Administration defines prognostic biomarkers as being able to "identify the likelihood of a clinical event or disease progression."[20] Therefore, prognostic biomarkers can augment clinical decision making, altering the therapies offered. Biomarkers have the added advantage of improving the understanding of the etiology of RF, which can be complex, particularly in the HCT population.

Currently, there is no simple blood test to guide the susceptibility to RF in the HCT recipient. Although some candidate proteomic biomarkers have been studied in the general adult population to predict the severity and mortality associated with acute respiratory distress syndrome (ARDS),[21,22] little data exist on biomarkers that can predict the development of RF, particularly in children. Because the HCT recipient is at high risk for RF, and many are already hospitalized at the time when critical illness develops,[23] they are an ideal population in which to develop a prognostic blood test, because early intervention may be possible. Therefore, we sought to identify novel biomarkers for RF, through a well-established quantitative tandem mass spectrometry–based proteomics discovery approach developed in our laboratory, by comparing plasma pooled from 15 patients with RF within 100 days post-HCT with plasma pooled from 15 patients without RF. We hypothesized that a panel of biomarkers detected as early as day 7 post-HCT can discriminate which patients are at high risk for RF and its related mortality. In addition, 2 markers (interleukin 6 [IL-6] and tumor necrosis factor receptor 1 [TNFR1]) were measured based on previous demonstrations of their involvement in idiopathic pneumonia syndrome (IPS) and nonrelapse mortality (NRM). Ultimately, these prognostic biomarkers for RF may allow for future innovative personalized therapies that will be more efficient if introduced early in the course of the disease in many patients post-HCT.

Patients and methods

Study population

Four cohorts (discovery, training, validation, and independent) of patients post-HCT were included in this study. The first 3 cohorts were taken from a larger HCT study of graft-versus-host disease consisting of 415 consecutive patients undergoing allogeneic HCT who were prospectively enrolled from 2013 through 2018 at 6 large US academic health centers: Indiana University School of Medicine, Johns Hopkins University School of Medicine, Texas Children's Hospital, Fred Hutchinson Cancer Research Center, Boston Children's Hospital, and Children's National Medical Center (#NCT02194439) (Figure 1).[24] The patient population was predominantly children, but it did include some adults. This study was approved by the 6 centers' institutional review boards, and patients were consented by trained study personnel. RF was defined as needing intubation and invasive mechanical ventilation for critical illness. Mortality with RF was defined as being intubated (within the first 100 days post-HCT) and dying while intubated or dying following a terminal extubation. An independent multicenter deidentified cohort of 48 children postallogeneic HCT was included. This was deemed exempt by the Indiana University Institutional Review Board. Because of the lack of detailed intubation information in this existing cohort, the outcome used for this analysis was admission to the ICU with the need for supplemental oxygen within 100 days post-HCT.

Proteomics workflow

Each sample was depleted of the 6 most abundant serum proteins using a Multiple Affinity Removal LC Column - Human 6 (Agilent). The depleted samples were subjected to protein precipitation using ice-cold acetone. Precipitated protein samples were resuspended in a solution of 8 M urea and 50 mM ammonium bicarbonate, reduced with dithiothreitol, alkylated with chloroacetamide, and digested with trypsin at a 1:25 enzyme/substrate ratio. The resulting peptide samples were desalted using Oasis C18 cartridges (Waters), dried by vacuum centrifugation, and labeled with tandem mass tags (Thermo Fisher Scientific). Labeled peptide samples were combined into 1 sample that was fractionated by basic reverse-phase chromatography, and pooled fractions were analyzed with an Orbitrap Fusion mass spectrometer. Mass spectrometry data were analyzed with Proteome Discoverer v2.2, using a human UniProt protein database that interrogates complex proteomes by matching mass spectra to a sequence database for protein identification. Results were filtered to a 1% false discovery rate at the peptide and protein levels.

Sample preparation and ELISA

Plasma samples were prospectively collected, frozen, and stored per institutional guidelines. Plasma samples were obtained at days 7 and 14 post-HCT, which were time points prior to the onset of RF in our discovery cohort (day +24 post-HCT). Proteins were measured in samples from the training and validation cohorts using commercially available enzyme-linked immunosorbent assays (ELISAs) and a sequential ELISA approach that was described previously.[25-27] Details are in supplemental Table 1. All samples and standards were tested in duplicate.

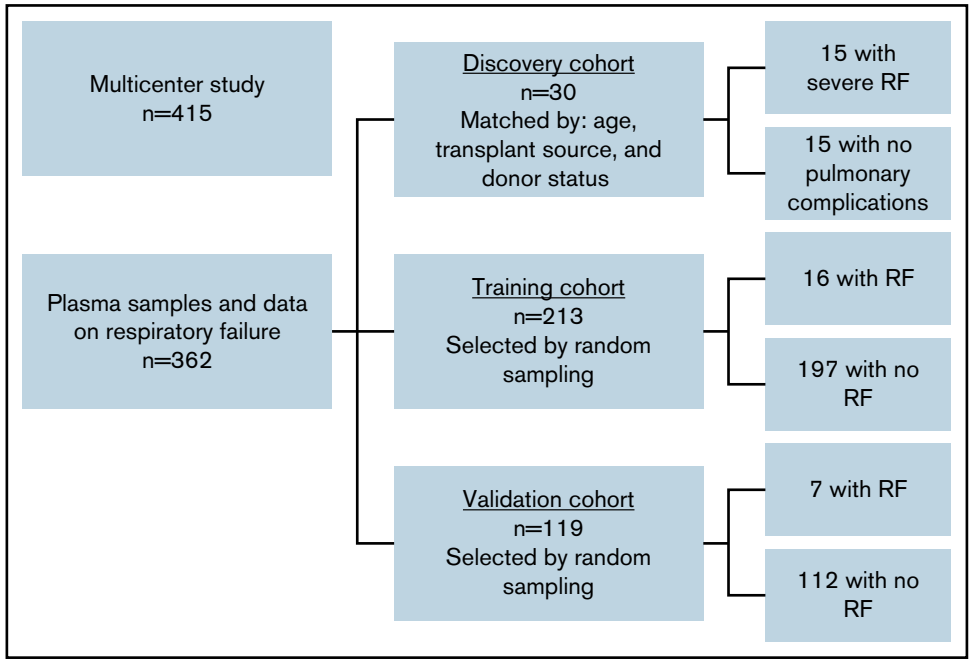

Figure 1. Workflow illustrating the study population and divisions into the discovery, training, and validation cohorts. The discovery cohort was selected using extreme phenotypes. The training and validation cohorts were made using a random 2/3 (training) and 1/3 (validation) selection process. RF defined as intubation (not for procedure) within the first 100 days post-HCT.

Statistical analysis

Discovery, training, and validation cohorts were created, and demographic characteristics were summarized. The discovery cohort was matched by age, donor status, and stem cell source. The training and validation cohorts were randomly generated from a multicenter cohort, resulting in a 2/3 split and a 1/3 split, respectively. Within the discovery cohort, median values of the 11 proteins were compared using a nonparametric Mann-Whitney *U* test, and those with a *P* value $\leq$ .05 were investigated further in the training and validation cohorts. Biomarkers were first analyzed as continuous variables using Cox proportional-hazards regression and subsequently dichotomized into high- and low risk-groups based on Youden's Index at day 14 to be more clinically relevant. Composite receiver operating characteristic curves were produced and compared to determine whether any combination of biomarkers offered better risk accuracy assessed by differences in the area under the curve (AUC). Kaplan-Meyer–based cumulative incidence and survival curves for RF by the biomarker level categories were produced and evaluated using log-rank tests at a 0.05 significance level. Landmark analysis, stacking day 7 and day 14, for the outcomes of RF and mortality with RF was also conducted.[28] All analyses were done using SAS 9.4. The 4 most promising biomarkers were tested on day 14 in the independent cohort using similar statistics described above.

Results

Demographics of the cohorts

Discovery cohort. We designed a discovery cohort comparing extreme RF phenotypes (severe RF vs no respiratory or other complications) within 100 days posttransplant using samples collected at day 14 post-HCT. The discovery cohort was matched between groups for age, donor status, and stem cell source. Demographics and transplant characteristics of the discovery cohort are in Table 1. By design, there was no difference in characteristics between those who developed RF and those who did not.

Training and validation cohorts. Using the multicenter biorepository of patients for whom we had RF information and biomarkers, we designed, by random sampling method of ~2/3 and 1/3, a training cohort of 213 patients (16 with RF and 197 without RF) and a validation cohort of 119 patients (7 with RF and 112 without RF). Patients without samples prior to intubation were excluded. Demographics of these 2 cohorts are provided in Table 1. Details about RF etiologies can be found in supplemental Table 2.

Independent cohort. A limited existing cohort of 48 children postallogeneic HCT for any indication with plasma samples available on day 14 post-HCT was used. Nine (18.8%) of the cohort experienced pediatric intensive care unit (ICU) admission with hypoxia, and 6 (12.5%) died within 180 days post-HCT. ICU transfer occurred at a median of 31 days (interquartile range [IQR], 24-45) post-HCT.

Proteomic biomarker discovery

We performed discovery proteomic analysis comparing plasma pooled from 15 patients with severe RF vs plasma pooled from 15 patients without RF or other complications (supplemental Figure 1). Plasma samples were taken at day 14 post-HCT, 10 days prior to the median onset of RF (24 days in the discovery cohort). Groups were matched for age, transplant source, and donor status, as described in the discovery cohort (Table 1). Of the 1106 proteins that were confidently quantified, 108 demonstrated a $\geq$1.25-fold

Table 1. Demographic and patient characteristics

	Discovery (N = 30)			Training (N = 213)			Validation (N = 119)		
Demographics	**RF (n = 15)**	**No RF (n = 15)**	***P***	**RF (n = 16)**	**No RF (n = 197)**	***P***	**RF (n = 7)**	**No RF (n = 112)**	***P***
Age, y	7.0 (1.0-18.0)	7.0 (3.0-19.0)	.512	9.0 (2.0-32.0)	12.0 (6.0-17.0	.674	15.0 (5-25.0)	12.5 (5.3-20.0)	.812
Sex									
Female	7 (46.7)	5 (33.3)	.456	9 (56.2)	82 (41.6)	.263	3 (42.9)	52 (46.4)	.999
Male	8 (53.3)	10 (66.7)		7 (43.8)	115 (58.4)		4 (57.1)	60 (53.6)	
White/non-Hispanic	11 (73.3)	11 (73.3)	.999	9 (56.3)	112 (56.9)	.976	3 (42.9)	68 (60.7)	.438
Malignant diagnosis	8 (53.3)	9 (60.0)	.713	12 (80.0)	121 (61.2)	.148	3 (42.9)	34 (30.4)	.376
Transplant source									
Marrow	3 (20.0)	3 (20.0)	.999	8 (50.0)	135 (68.5)	.328	5 (71.4)	60 (54.1)	.088
Cord/double cord	9 (60.0)	9 (60.0)		3 (18.8)	22 (11.2)		2 (28.6)	12 (10.8)	
PBSCs	3 (20.0)	3 (20.0)		5 (31.2)	40 (20.3)		0 (0)	39 (35.1)	
Related donor	2 (13.3)	5 (20.0)	.999	10 (62.5)	99 (50.3)	.367	5 (71.4)	53 (47.3)	.264
HLA matched	9 (60.0)	7 (46.7)	.464	12 (75.0)	154 (82.8)	.494	6 (85.7)	86 (79.6)	.999
Conditioning intensity									
Myeloablative	11 (73.3)	12 (80.0)	.999	11 (68.8)	144 (73.1)	.772	2 (28.6)	78 (69.6)	.037
Reduced intensity	4 (26.7)	3 (20.0)		5 (31.3)	53 (26.9)		5 (71.4)	34 (30.4)	
Total body irradiation	5 (33.3)	5 (33.3)	.999	9 (56.3)	82 (41.6)	.255	5 (71.4)	47 (42.0)	.237
Acute graft-versus-host disease*	6 (40.0)	6 (40.0)	.999	5 (31.3)	60 (30.5)	.999	1 (14.3)	41 (36.6)	.419
Veno-occlusive disease*	1 (6.7)	4 (26.7)	.330	1 (6.3)	9 (4.6)	.550	1 (14.3)	5 (4.5)	.311
Time to onset of RF, median (IQR), d	24 (9-30)			19 (11-26)			14 (9-20)		

Unless otherwise noted, data are n (%). Continuous variables were compared using the Mann-Whitney *U* test, and categorical variables were compared using the χ^2 test or Fisher's exact test, where appropriate. PBSC, peripheral blood stem cell.

*These variables were evaluated up to the development of RF or 180 days, whichever came first.

Table 2. HRs for the training and validation cohorts with biomarkers dichotomized by Youden's index

	Training cohort		Validation cohort	
Day-14 biomarker	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Unadjusted model				
ST2 ≥ 54.5 ng/mL	4.5 (1.6-12.5)	.004	17.5 (2.1-145.7)	.008
OPN ≥ 381.5 ng/mL	9.1 (3.1-26.7)	<.001	2.3 (0.5-10.4)	.271
WFDC2 ≥ 61.2 ng/mL	4.2 (1.4-12.4)	.008	14.0 (3.1-63.0)	.001
SFTPB ≥ 1219.7 ng/mL	5.0 (1.6-15.6)	.006	4.1 (0.8-21.9)	.091
IL-6 ≥ 77.5 pg/mL	6.9 (2.4-20.2)	<.001	9.0 (1.7-46.1)	.009
TNFR1 ≥ 6237.0 pg/mL	6.2 (2.2-17.0)	<.001	18.1 (3.5-93.4)	.001
Multivariable model adjusting each individual biomarker for conditioning regimen				
ST2 ≥ 54.5 ng/mL	4.5 (1.6-12.5)	.004	23.2 (2.8-195.5)	.013
WFDC2 ≥ 61.2 ng/mL	4.2 (1.4-12.4)	.010	18.2 (3.9-83.9)	.019
IL-6 ≥ 77.5 pg/mL	6.9 (2.3-20.1)	<.001	12.2 (2.4-64.1)	.014
TNFR1 ≥ 6237.0 pg/mL	6.1 (2.2-16.9)	<.001	16.1 (3.1-83.5)	.001

CI, confidence interval; OPN; osteopontin; SFTPB, surfactant protein B.

increase in the tandem mass tag duplex label. From the identified proteins, we selected 11 proteins for further analysis based on pathway networks, published literature, and ELISA availability (supplemental Table 3). Biologic plausibility was determined with an in-depth review of gene information using PubMed (https://www.ncbi.nlm.nih.gov/gene) and a review of published literature in OVID. Intracellular proteins were excluded, and the availability of reliable commercially available ELISA kits was determined for the remaining proteins. Mass spectrometry is limited in its ability to reliably detect low-abundance proteins. Therefore, as has been done previously,[29] we also measured IL-6 and TNFR1 based on their previous association with IPS[30] and NRM.[24]

The 13 candidate proteins (11 discovered plus IL-6 and TNFR1) were tested with ELISAs in individual plasma samples from the discovery cohort. The median and IQRs of these proteins were compared for patients who did and did not develop RF (n = 15 each) (supplemental Table 4). Six candidates with a *P* value ≤ .05 were chosen for further investigation: Stimulation-2 (ST-2; the IL-33 decoy receptor), osteopontin, WAP 4-disulfide core domain protein 2 (WFDC2; also known as human epididymis 4), surfactant protein B, IL-6, and TNFR1.

Development of a biomarker panel for RF at day 14 post-HCT

Using sequential ELISAs, the levels of the 6 identified candidate biomarkers were measured in plasma from the training (n = 213) and validation (n = 119) cohorts described above (demographics are shown in Table 1). The biomarkers were first explored as continuous variables to determine their association with the development of RF. A standard deviation incremental increase in all 6 biomarkers was associated with an increased risk for the development of RF by 100 days post-HCT in the training cohort and was replicated in the validation cohort (supplemental Table 5).

To improve the clinical applicability of the model, biomarkers were dichotomized into high- and low-risk groups based on whether they were above or below the cut-point determined by Youden's index. Four biomarkers (ST2, WFDC2, IL-6, and TNFR1) were validated in multivariable analysis (Table 2). Sensitivity, specificity, and positive and negative predictive values can be found in supplemental Table 6. After adjusting for the intensity of the conditioning regimen in a multivariable model, elevated levels of all 4 biomarkers remained significant in the training and validation cohorts (Table 2). High levels of these 4 biomarkers on day 14 post-HCT were associated with a greater cumulative incidence of RF (training cohort, Figure 2A-D; validation cohort, Figure 2E-H). Because the onset of RF was variable and close to day +14 in the training and validation cohorts, an independent cohort was also tested with a median day to outcome of 31 days. In this cohort, ST2 ($P = .028$) and TNFR1 ($P = .003$) were statistically significant using the same biomarker cut-points. WFDC2 and IL-6 had increased hazard ratios (HRs), but did not quite reach statistical significance (supplemental Figure 2; supplemental Table 7). WFDC2 was likely limited by the variability of biomarker levels between cohorts and the optimal cut-point derived from the training cohort (supplemental Figure 3).

Combinations of these 4 biomarkers were explored using the AUC of the composite receiver operating characteristic curve. All 4 biomarkers demonstrated the ability to discriminate patients who developed RF within 100 days post-HCT from those who did not, with AUCs of 0.61 to 0.72 in the training cohort and 0.72 to 0.87 in the validation cohort. The combination of all 4 biomarkers into a composite panel increased the AUC to 0.75 in the training cohort and to 0.90 in the validation cohort (supplemental Figure 4), although these improvements in AUC did not reach statistical significance. Of note, the AUC was similar for all 4 biomarkers in the independent cohort (supplemental Table 8).

Landmark analysis of biomarkers at days 7 and 14 post-HCT as predictors of RF development

Because 8 patients had samples taken close to the onset of RF, we measured the 4 biomarkers (ST2, WFDC2, IL-6, and TNFR1) at day 7 post-HCT. AUCs for biomarkers on day 7 ranged from 0.58 to 0.74 in the training cohort and from 0.65 to 0.96 in the validation cohort (supplemental Table 9). Day-7 and day-14 biomarker values were stacked and assessed for the risk of RF using a

cause-specific landmark analysis. With this landmark analysis, high levels of these 4 biomarkers measured as early as day 7 post-HCT were associated with an increased risk for developing RF within the first 100 days post-HCT in the training and validation cohorts (Table 3; supplemental Figure 5).

Landmark analysis of biomarkers as predictors for mortality with RF

Next, we examined the ability of ST2, WFDC2, IL-6, and TNFR1 on days 7 and 14 post-HCT to predict mortality with RF. The 3 cohorts used were the same as those described above. Mortality with RF rates in the various cohorts were as follows: discovery cohort 20% (6/30), training cohort 4% (8/213), and validation cohort 3% (4/119).

Because the day-14 median levels of of ST2, WFDC2, IL-6, and TNFR1 were statistically higher for those who died compared with those who survived in the discovery cohort (supplemental Table 10), we evaluated them as predictors of an increased risk for mortality with RF. Cause of death is listed in supplemental Table 11. Only ST2, WFDC2, and TNFR1 were found to be significant in the training and validation cohorts (supplemental Table 12). We next categorized the biomarkers into high- and low-risk groups using the same cut-point as for the risk of developing RF. On day 14 post-HCT, high-risk biomarkers were associated with worse mortality with RF (supplemental Figure 6) and NRM within a year post-HCT (Figure 3). We then performed a landmark analysis to evaluate the combination of day-7 and day-14 biomarkers categorized into high- and low-risk groups using the same cut-point as for the risk of developing RF. Even as early as day 7 post-HCT, ST2, WFDC2, and TNFR1 predicted an increased risk of mortality with RF (Table 4).

Discussion

In 3 cohorts of patients post-HCT, high day-14 levels of ST2, IL-6, and TNFR1, as well as newly identified WFDC2, were identified and validated as biomarkers associated with the development of RF within the first 100 days post-HCT. An independent pediatric cohort also demonstrated significance

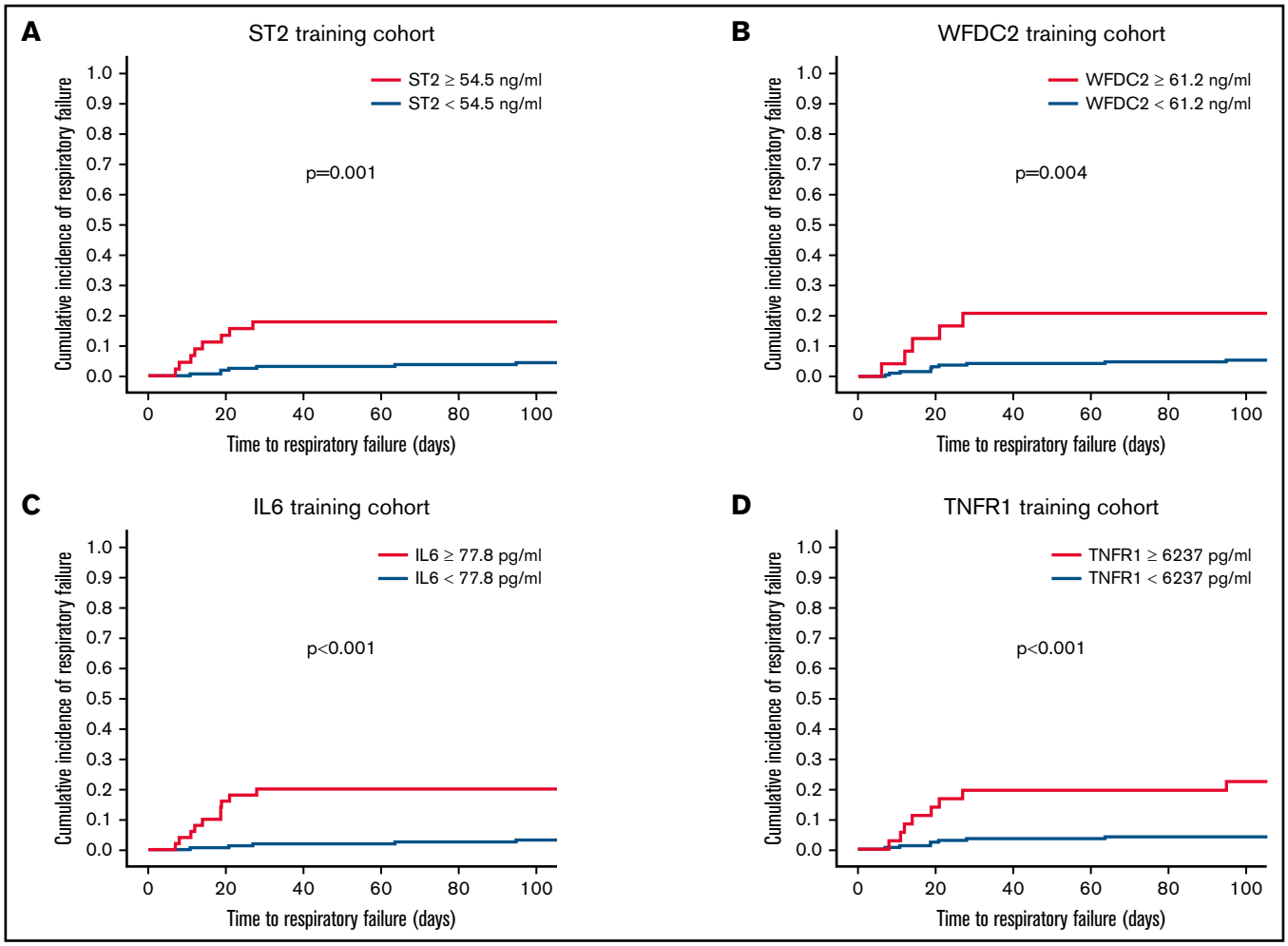

Figure 2. Cumulative incidence functions of the training and validation cohorts. A higher cumulative incidence of the development of RF for those with high biomarker levels (red line) is seen on day 14 post-HCT compared with those with levels below the cut-point chosen by Youden's index (blue line). High levels of ST2, WFDC2, IL-6, and TNFR1 predicted a statistically significant increase in the development of RF within 100 days posttransplant.

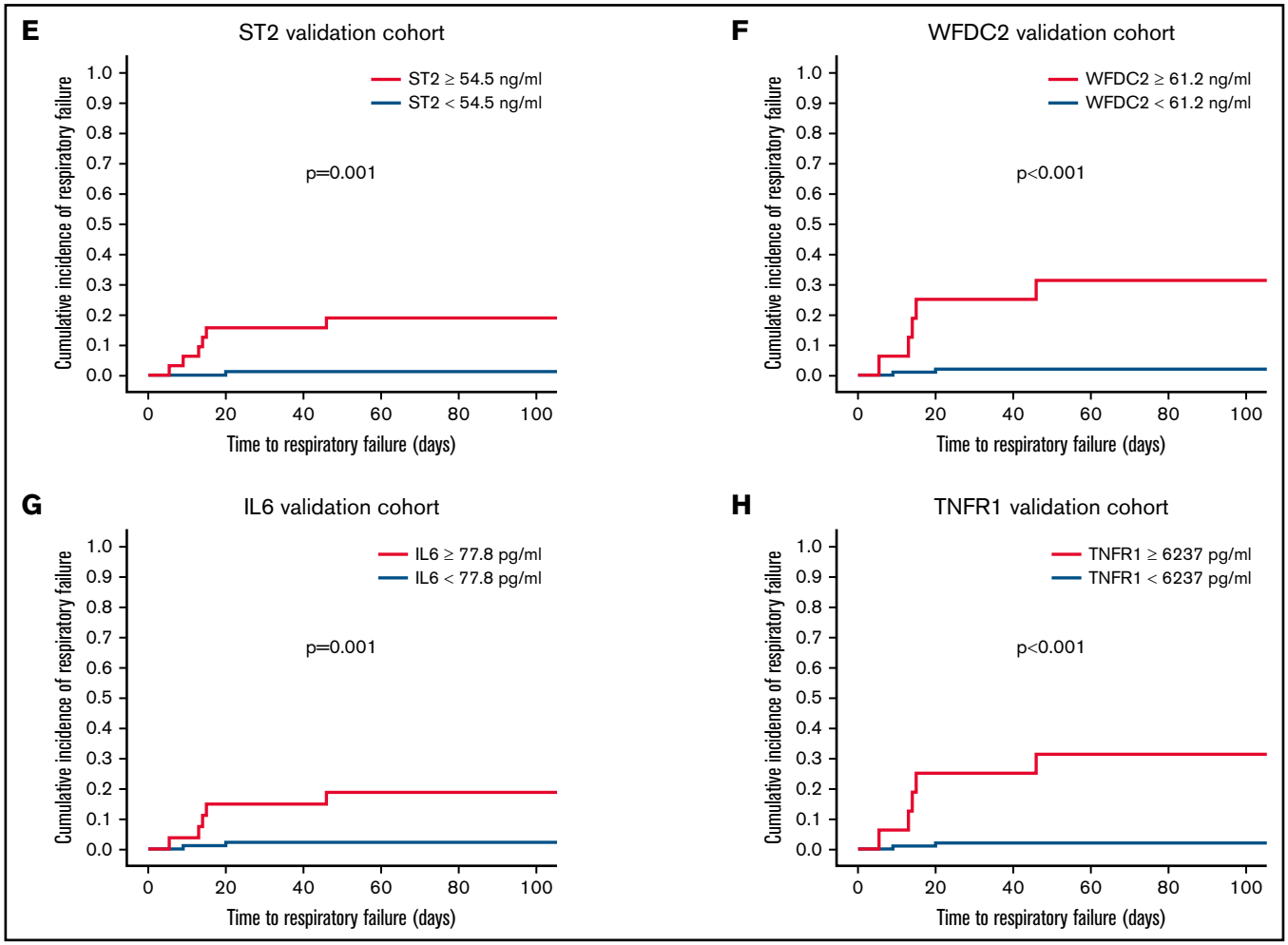

Figure 2. (Continued).

for increased day-14 levels of ST2 and TNFR1, as well as great potential for WFDC2 and IL-6, for admission to the ICU with hypoxia. On landmark analysis, these proteins also demonstrated promise as risk biomarkers as early as day 7 post-HCT. Furthermore, increased levels of ST2, WFDC2, and TNFR1 were early predictors of mortality with RF. The use of biomarkers that herald the development of RF may allow for early detection, therapeutic intervention, and improved preventative counseling.

Although the mortality rate in the intubated HCT recipient has improved,[31] the historical fear still remains, causing HCT physicians to question the appropriate time to involve the critical care team and intensivists in the struggle over when/whether to intubate. This may lead to a delay in intervention, resulting in missed opportunities for aggressive and early management. Delay in critical care is associated with a higher mortality.[8,32] Multicenter studies suggest that the longer a patient spends in the ICU or on supplemental O_2 prior to intubation, the higher

Table 3. Landmark HRs for the training and validation cohort with high biomarkers levels on days 7 and 14 post-HCT for the development of RF

	Training cohort		Validation cohort	
Biomarkers at days 7 and 14	**Landmark HR (95% CI)**	***P***	**Landmark HR (95% CI)**	***P***
ST2 ≥ 54.5 ng/mL	3.49 (1.39-8.76)	<.001	6.03 (1.61-22.65)	<.001
WFDC2 ≥ 1.2 ng/mL	3.08 (0.84-11.3)	.09	14.21 (3.36-60.04)	<.001
IL-6 ≥ 77.5 pg/mL	5.32 (2.48-11.40)	<.0001	4.58 (1.05-20.05)	.04
TNFR1 ≥ 6237.0 pg/mL	3.83 (1.43-10.28)	<.001	22.52 (4.03-126.05)	<.001

CI, confidence interval.

the mortality.[8,33] Further, a recently published multicenter study found an unprecedented 10% cardiac arrest rate during intubation of patients post-HCT, suggesting a possible delay in definitive care.[12] Objective data, such as a susceptibility biomarker, may aid in these complex and difficult decisions.

Although limited by the numbers of cases, this study demonstrates that early measurements of ST2, WFDC2, IL-6, and TNFR1 are associated with RF. This noninvasive and objective risk assessment may complement the clinical evaluation of HCT recipients. Although the positive predictive value (PPV) is low in our cohort, this is likely a reflection of the low prevalence of RF. The use of PPV for biomarkers of complications post-HCT is debatable, and sensitivity has been suggested as a more useful statistic.[34] Despite the low PPV, these biomarkers can still be meaningful in a life-threatening complication such as RF, because we would like to capture all cases of RF; increased numbers of false negatives would not be a problem because the treatment proposed here is increased monitoring and stricter use of the recommended guidelines. Indeed, the implications for patients are that if all patients are screened with these biomarkers early in the transplant course, those with the highest risk could have stricter surveillance for signs of respiratory distress and potential implementation of standard therapies, such as aggressive diuresis, preemptive transfer to an intensive care setting, early initiation of noninvasive respiratory support, and avoidance of a delay in intubation, as have been suggested by many multicenter studies.[8,12,35,36] Further, in prospective studies, the PPV and clinical utility could be improved if combined with known prognostic clinical variables, such as transplantation from an unrelated donor,[12] and the need for >1 L of supplemental oxygen, weight gain, and early warning scores for critical illness in the peri-HCT period.[16,37,38] Future investigations should focus on incorporating plasma biomarkers with clinical risk factors for RF.

Identification of the risk for RF in the HCT population may allow for earlier personalized interventions. Targeted therapy of IPS with etanercept (TNF inhibitor) has demonstrated success,[39] and our data suggest that combinatorial strategies targeting other proteins identified herein may further improve outcomes. Moreover, earlier supportive therapies may be undertaken at the first sign of respiratory

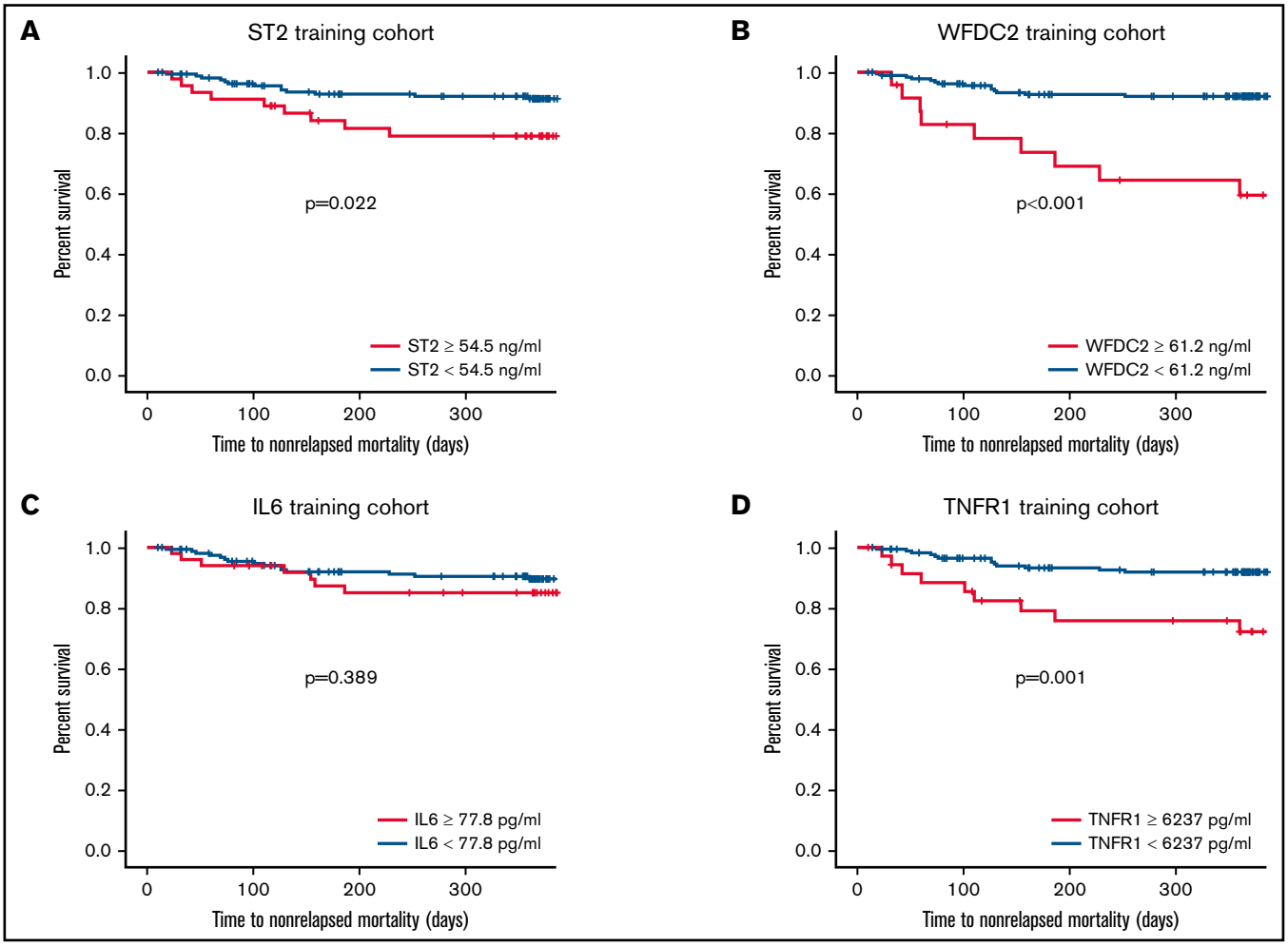

Figure 3. Survival curves for NRM mortality at 1 year stratified by high/low biomarker levels. Patients with high biomarker levels (red line) on day 14 post-HCT vs with those with levels below the cut-point (blue line); the cut-point based on Youden's index was used for this analysis. In the training cohort, high levels of ST2, WFDC2, and TNFR1 were statistically significantly associated with NRM. In the validation cohort, high levels of 4 biomarkers predicted a statistically significant increase in NRM.

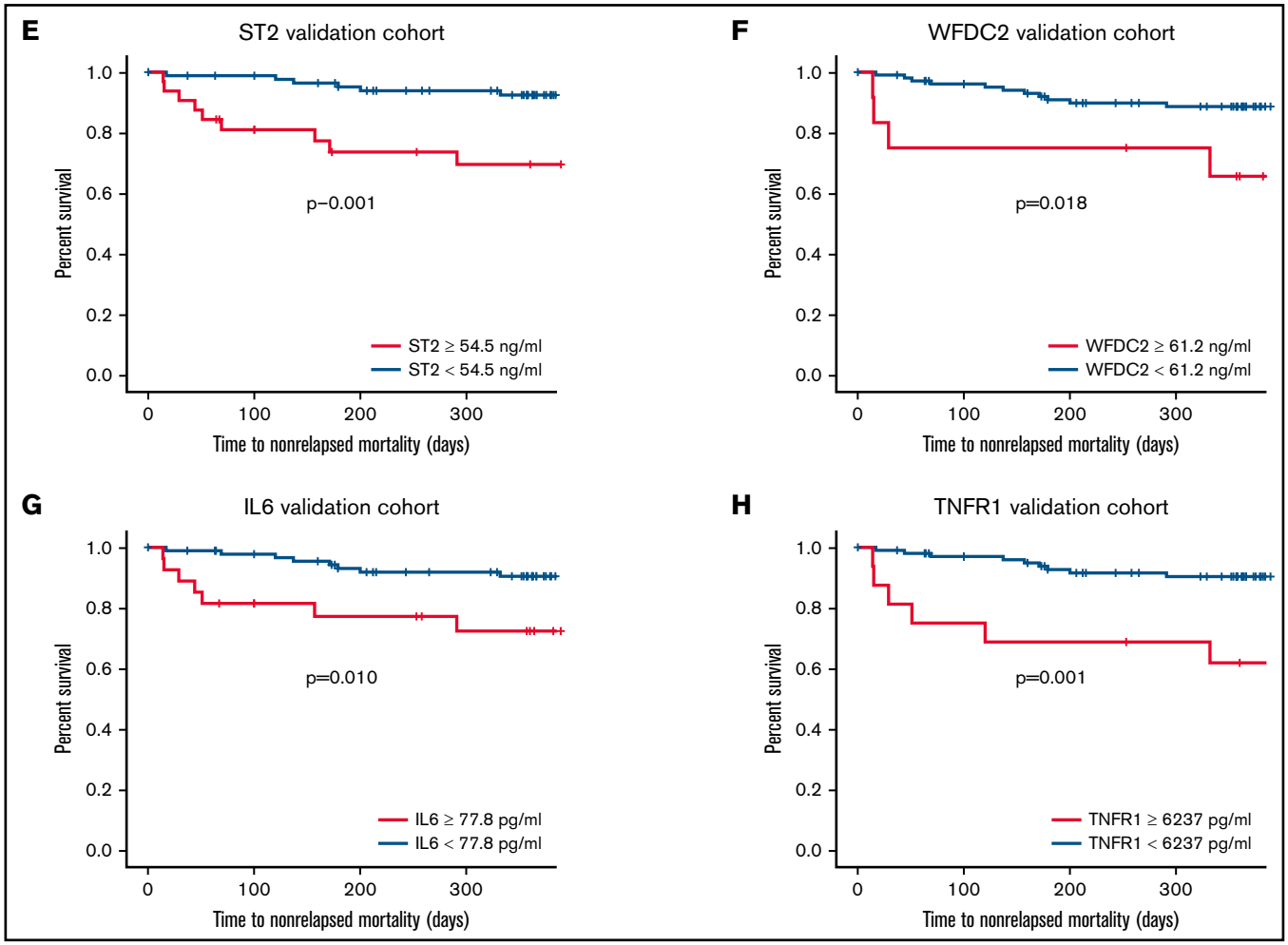

Figure 3. (Continued).

distress in patients identified as particularly vulnerable to developing RF. Clearly, any toxicity from these novel therapies would need to be thoroughly investigated prior to the widespread use of biomarker-driven therapy. Another future approach is to develop a preemptive therapeutic intervention based on high-risk biomarkers, shifting critical care interventions from reactionary to preventative in this high-risk population.

The cause of RF can be challenging to ascertain, because post-HCT, patients are at risk for infectious[40-42] and noninfectious pulmonary complications.[43] These biomarkers may help to unravel the etiology of RF in this population. It is possible that a common inflammatory pathway leads to the development of severe lung injury in this population. Complementing the findings reported herein, we showed previously that components of the acute-phase response

Table 4. Landmark HRs for the training and validation cohort with high biomarkers levels on days 7 and 14 post-HCT for the outcome of mortality with RF

	Training Cohort		Validation cohort	
Biomarkers at days 7 and 14	**Landmark HR (95% CI)**	***P***	**Landmark HR (95% CI)**	***P***
ST2 ≥ 54.5 ng/mL	3.91 (1.11-13.75)	.03	20.63 (3.1-138.3)	.001
WFDC2 ≥ 61.2 ng/mL	4.81 (0.82-28.2)	.08	9.69 (2.4-39.5)	.002
IL-6 ≥ 77.5 pg/mL	5.21 (2.17-12.51)	<.001	6.23 (1.2-30.5)	.02
TNFR1 ≥ 6237.0 pg/mL	7.20 (1.83-28.28)	<.004	49.03 (5.5-439.1)	<.0001

CI, confidence interval.

signaling pathway involving TNF-α and IL-6 are operative in the development of acute noninfectious lung inflammation after HCT.[44] From a clinical perspective, these patients are at high risk for the development of ARDS.[45] In fact, >90% of intubated children develop ARDS during the first week of ventilation post-HCT.[46] It remains to be determined whether these biomarkers signal a common inflammatory pathway that leads to the development of RF or identify specific molecular pathways that are responsible for lung injury. Although preclinical data support the latter, further study is clearly needed.

These biomarkers may lead to future novel targeted therapeutic options, resulting in improved outcomes. ST2, the IL-33 receptor, is a mediator for inflammation.[25,47] IL-6, a proinflammatory cytokine, activates T cells and induces synthesis of acute-phase proteins.[39] TNFR1 is involved in the inflammatory response.[22] Therapeutics targeting these 3 biomarkers are being used or tested for complications of cellular therapies. Etanercept is already well accepted in the pediatric HCT population as a therapy for IPS,[39] altering the care of this disease that previously had very few therapeutic options. Tocilizumab targets IL-6 and is used for graft-versus-host disease prophylaxis and in the cytokine storm associated with engineered T-cell therapy.[48,49] Finally, anti-ST2 exists as an antibody and as a small molecule. It was proved to be effective in experimental models of IPS and graft-versus-host disease.[50,51] WFDC2, also known as human epididymis 4, has been investigated only minimally, particularly in RF. It is a member of the whey acid protein family. Secretory leukocyte protease inhibitor and elafin are also members of this family and have been studied more extensively. These proteins are believed to be stimulated by proinflammatory mediators and have antiproteinease and antibacterial properties[52-56]. Elevated levels of elafin are associated with skin graft-versus-host disease.[56] Specific to WFDC2, it is expressed in pulmonary epithelial cells and may have a role in innate immunity of the respiratory system, and its expression may be altered in pulmonary disease.[57] WFDC2 may represent a novel target for therapeutic development. It was not statistically significant in the independent cohort, which was likely related to the sample size and variability in the distribution of biomarker levels among cohorts. This novel marker deserves further investigation for additional validation, as well as identification of the optimal clinically relevant cut-points.

This study has some limitations, 1 of which is the number of patients with RF as the outcome; this is reflected in the width of our confidence intervals. A larger prospective cohort may be needed to add to the validity of these findings. The small sample of patients with RF precluded additional analyses for particular causes of RF, which is important when evaluating specific therapies. However, even with the small sample sizes, we were able to demonstrate statistical significance, and the use of separate discovery, training, and validation cohort adds strength to the significance of our study. Our mass spectrometry approach has inherent limitations, particularly the ability to capture low-abundance proteins.[58] We accounted for this by including 2 additional markers that were shown to be important in pulmonary complications in this population. Another limitation is the inclusion of pediatric and adult HCT patients in a single analysis, because of the possible different disease processes and approaches to critical care management. Moving forward with future study designs, it would be important to investigate these patient populations separately. An additional limitation is that these biomarkers may not be specific for RF, because it is incredibly complex in this population; however, these markers may be more specific for alloreactivity or other underlying causes of RF. Using proteomics to better understand the biology of RF in this population would allow for more directed implementation of standard therapies, such as etanercept for IPS. This is an important focus for further investigation.

In conclusion, high levels of ST2, WFDC2, IL-6, and TNFR1 measured as early as day 7 post-HCT are associated with the development of RF within the first 100 days post-HCT and with mortality with RF. These biomarkers offer objective data to begin to identify the highest-risk patients who may benefit from early intervention; they may also hold promise for therapeutic targets to alter the course and outcome of RF.

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Authorship

Contribution: C.M.R. and S.P. conceived, designed, and oversaw the study and interpreted the results; C.M.R., A.M., and S.P. developed the data collection forms, built and maintained the database, and ensured data integrity. F.P. and C.M.R. analyzed data; C.N.D., R.A.K., P.A.C., D.J., C.M.B., C.R.Y.C., S.F., J.R., and K.R.C. oversaw patient sample collection and processing at the respective centers; C.M.R., M.R.L., and S.P. were responsible for centralized sample processing, storage, and proteomic analysis with ELISAs; and P.R.G. conducted the discovery proteomic analyses and interpreted the results. All authors reviewed and provided feedback on the initial study design, collected data, interpreted results, and read, edited, and approved the final version of the manuscript.

Conflict-of-interest disclosures: C.N.D. is a consultant for Advanced Clinical and Novartis. C.R.Y.C. is a cofounder of Mana Therapeutics, a biotech company focused on developing T-cell therapies for cancer, and is a consultant for Catamaran Bio, a biotech company developing natural killer cell therapies for cancer (with

intellectual property on both platforms). K.R.C. has received grant support from and is a consultant, member of the advisory board, and educational speaker for Jazz Pharmaceuticals and is on the advisory board and is a consultant for Mesoblast. S.P. is an inventor on a patent on "Methods for detecting graft-versus-host disease" (US13/573,766). The remaining authors declare no competing financial interests.

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