Figure 1. High-resolution computed tomography of the chest (Case 3), consistent with silicosis with progressive massive fibrosis, complicated by pneumothorax.

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Marianne T. Turner, M.B. B.S., B.A., M.P.H.
St. Vincent's Hospital
Darlinghurst, Australia

Sameh R. Samuel, M.B. B.S.
Wollongong Hospital
Wollongong, Australia

Elizabeth J. Silverstone, M.B. B.S.
Deborah H. Yates, B.A., M.A., M.B. B.Chir., M.Sc., Dip. Occ. Med.
(L.S.H.T.M.), M.D. (Cantab.)*
St. Vincent's Hospital
Darlinghurst, Australia

*Corresponding author (e-mail: d.yates@unsw.edu.au).

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Markers of Endothelial Dysfunction and Cytokines in High-Risk Pediatric Patients with Severe Sepsis

To the Editor:

Inflammatory cytokines and biomarkers of endothelial dysfunction have been associated with disease severity and mortality in severe sepsis (1–5). Through the application of biomarkers, treatment of sepsis has begun to transition from “one-size-fits-all” supportive therapy to individualized or personalized treatment based on patient-specific endotypes (6).

As a result of heterogeneity within patient populations, some subgroups may benefit from a specific treatment while others may be harmed by the same therapy (7). This is of utmost importance

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with regard to pediatric populations because of the inherent developmental differences that occur as children age. Although the identification of patient endotypes and phenotypes may lead to improved patient outcomes, this has been largely unfeasible in critical illness because the time required to perform biomarker tests makes identification of endotypes impractical. Thus, investigators in critical care have been limited in implementing a precision medicine approach in clinical trials in part because of the inability to measure protein biomarkers in real time (8).

In this proof-of-concept study, we applied a validated multibiomarker-based prognostic algorithm to identify patients at high risk of mortality and quantified levels of markers of inflammation and endothelial dysfunction to illustrate the feasibility of identifying prognostic and predictive enriched cohorts of pediatric patients with severe sepsis. In addition, we validated a novel microfluidic device for rapid identification of plasma proteins as a proof of concept for future biomarker-driven adaptive clinical trials. Some of the results of this study have been previously reported in the form of an abstract (9).

Methods

Plasma samples were obtained within 48 hours of a diagnosis of severe sepsis (10) in patients 0–17 years of age, between 2012 and 2016, at C.S. Mott Children's Hospital (Ann Arbor, Michigan). Informed consent was obtained from the parent or guardian of all participants for this institutional review board-approved protocol.

Levels of the Pediatric Sepsis Biomarker Risk Model (PERSEVERE) (11) risk-stratification biomarkers GZB (granzyme B), HSPA1B (heat shock protein family A member 1B), IL-8,

CCL-3 (c-c motif chemokine ligand 3), and MMP-8 (matrix metalloproteinase 8), and concentrations of the cytokines IL-1 β , IL-6, IL-7, IL-10, IL-13, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), IFN- γ , and TNF- α (tumor necrosis factor- α) were determined in triplicate using the Bio-Rad multiplex suspension array system. Plasma Ang-1 (angiopoietin-1) and Ang-2 were measured using commercially available ELISA kits (R&D Systems, Inc.). VWF (von Willebrand factor) and VWFpp (VWF propeptide) were measured using the AlphaLISA assay (PerkinElmer) (12). All protein measurements were conducted on samples drawn at the same time point.

Plasma cytokine and endothelial marker concentrations were also determined using the MicroKine biomarker assay system (PreDxion Bio, Inc.), which leverages localized surface plasmon resonance-based biosensors to provide label-free, rapid, multiplex, protein biomarker detection (13). Plasma (5 μ l) was loaded into a custom (IL-8, CCL-3, MMP-8, GZB, HSPA1B, IL-6, TNF- α , Ang-1, and Ang-2) assay cartridge and incubated at room temperature for 20 minutes. Channels were washed using phosphate-buffered saline. The cartridges were read via the MicroKine Assay Reader, and biomarker values were determined in triplicate with internal standard curves and quality controls.

Comparisons between high-risk and low-risk cohorts, as stratified by PERSEVERE, were performed using tests specified in Table 1. The assay values were log transformed and the relationship between levels of plasma biomarkers determined by standard assays (Bio-Plex, ELISA, and AlphaLISA) and the MicroKine system were evaluated using Bland-Altman plots and linear regression (Prism v8.0.1; GraphPad Software Inc.).

Table 1. Patient Characteristics

Parameter	All Patients (n = 33)	High-Risk Cohort (n = 8)	Low-Risk Cohort (n = 25)	P Value
Age, yr, median (IQR)	9.8 (1.3–13.2)	8.7 (2.7–13.9)	9.8 (1.0–13.1)	0.82*
Male, n (%)	20 (60.6)	4 (50.0)	16 (64.0)	0.68†
PRISM 3 score, median (IQR)	11 (6–16)	20 (16–31)	9.5 (6–12)	<0.001*
Comorbidity, n (%)	22 (66.7)	5 (62.5)	17 (68.0)	0.77†
Hospital LOS, d, median (IQR)	16.0 (9.0–18.0)	14.0 (4.8–45.5)	16.0 (9.0–22.5)	0.96*
ICU LOS, d, median (IQR)	10.0 (6.0–19.5)	9.0 (4.0–36.5)	11.0 (6.0–17.0)	0.89*
Mechanical ventilation, n (%)	31 (93.9)	8 (100.0)	23 (92.0)	0.41†
Mortality, n (%)	6 (18.2)	6 (75.0)	0 (0.0)	<0.001*
Analyte, pg/ml, median (IQR)				
IL-1 β	0.0 (0.0–1.2)	2.5 (0.5–20.6)	0.0 (0.0–0.5)	0.001*
IL-6	36.5 (10.3–234.7)	2,958 (76–10,724.4)	25.2 (5.95–54.3)	0.003*
IL-7	0.0 (0.0–3.1)	4.6 (0.8–8.2)	0.0 (0.0–0.0)	0.001*
IL-10	22.9 (1.9–203.3)	285.7 (152.0–482.5)	13.3 (0.5–73.1)	<0.001*
G-CSF	349.4 (98.6–4,836.1)	20,574 (1,124.8–21,569.1)	245.8 (69.1–748.9)	<0.001*
GM-CSF	2.74 (0.0–14.7)	40.5 (7.5–914.6)	1.6 (0.0–6.6)	<0.001*
IFN- γ	5.5 (0.3–45.4)	43.4 (5.4–409.3)	2.1 (0.0–28.1)	0.06*
TNF- α	15.1 (9.5–31.8)	74.2 (23.3–293.7)	12.6 (8.3–20.3)	0.003*
Ang-2	5,451.4 (3,468.0–18,109.3)	76,625.0 (42,747.3–82,894.8)	4,456.1 (2,846.3–10,444.6)	<0.001*
Ang-1	484.2 (212.4–771.2)	656.1 (596.7–962.1)	381.8 (202.9–720.5)	0.21*
VWF	82.2 (63.3–141.9)	156.9 (133.1–202.4)	80.9 (56.6–113.8)	0.004*
VWFpp	165.0 (108.3–342.9)	649.6 (496.6–720.6)	131.4 (104.0–194.2)	<0.001*

Definition of abbreviations: Ang-1 = angiopoietin-1; Ang-2 = angiopoietin-2; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage CSF; IQR = interquartile range; LOS = length of stay; PRISM 3 = Pediatric Risk of Mortality 3; TNF- α = tumor necrosis factor- α ; VWF = von Willebrand factor; VWFpp = VWF propeptide.

*Mann-Whitney *U* test.

†Fisher's exact test.

Results

Thirty-three patient samples were available for analysis. Clinical characteristics of the patients are shown in Table 1. Using the PERSEVERE risk-stratification model, we stratified 4 patients as having a high risk of mortality, 4 as having an intermediate risk, and 25 as having a low risk. Because our intent was to demonstrate the feasibility of separating patients who had a low risk of mortality with current care and patients with a higher risk of mortality who might receive intervention in a clinical trial, the PERSEVERE high- and intermediate-risk groups were grouped and defined as high-risk ($n = 8$).

Median cytokine levels were significantly elevated in the high-risk cohort compared with the low-risk cohort, with the exception of IL-13 and IFN- γ (Table 1). Similarly, Ang-2, VWF, and VWFpp were significantly elevated in the high-risk group compared with the low-risk group (Table 1). There was no significant difference, however, in median Ang-1 levels between the two patient groups.

The MicroKine biomarker detection assay platform quantified levels of GZB, IL-8, MMP-8, HSPA1B, Ang-1, Ang-2, IL-6, and TNF- α . The MicroKine quantification results were highly correlated with results obtained using standard immunoassay platforms with a

Figure 1. Measurement of biomarkers of inflammation and endothelial dysfunction (Ang-2 [angiopoietin 2], IL-6, and TNF- α [tumor necrosis factor- α]) using a standard assay and the MicroKine Biomarker Assay System. (A) Standard ELISA results, showing increased biomarker levels and heterogeneity in patients with severe sepsis and an increased risk of mortality. Solid circles represent survivors and red X marks represent nonsurvivors. (B) Correlation between a commercially available immunoassay platform (e.g., multiplex-bead-based assay [MBBA]) or ELISA (on the x-axis) and measurements obtained using the MicroKine biomarker assay system (on the y-axis). (C) Bland-Altman plots demonstrate agreement between the MicroKine assay and ELISA. The horizontal solid line represents the mean difference between the methods, and the dotted lines represent ± 1.96 SD.

coefficient of determination (R^2) between 0.936 and 0.992 (Figure 1). Bland-Altman comparisons demonstrated the following mean differences: GZB -1.69 (confidence interval [CI], -6.55 to 3.18), IL-8 7.2 (CI, -81.4 to 95.8), MMP-8 -116.5 (CI, -7.5×10^{-3} to $7.311.2$), HSPA1B $2,230.2$ (CI, -1.0×10^5 to 1.1×10^5), Ang-1 -55.64 (-449.4 to 338.1), Ang-2 2.89 (CI, -14.0 to 19.8), IL-6 11.4 (CI, -66.3 to 89.1), and TNF- α 3.78 (CI, -6.0 to 13.6).

Discussion

Our data prospectively demonstrate the proof of concept that rapid biomarker detection is feasible in critically ill children, and with this, the possibility of prognostic and predictive enrichment. In addition, the results provide further evidence of endothelial dysfunction in severe sepsis, consistent with previous studies that demonstrated an association between mortality and Ang-2 (2–4). Similarly, our findings are consistent with a previous study that showed an association between cytokines and organ dysfunction and mortality in sepsis (14). The PERSEVERE risk-stratification tool continues to demonstrate its ability to stratify patients, as all of the deaths in our cohort occurred in patients who were defined as having a high or intermediate risk. We also validated a novel microfluidic assay that can accurately and quickly measure plasma biomarkers, and demonstrated the feasibility of rapid prognostic enrichment and identification of predictive therapeutic targets in critically ill patients. Combining prognostic and predictive enrichment strategies can optimize future patient selection, targeting specific therapies to high-risk patients, as was previously suggested in a secondary analysis of steroid use in septic shock (6).

The small sample size in this preliminary study, especially the sample of patients at increased risk for poor outcomes, necessitates analysis of larger patient cohorts to confirm these findings. Additional limitations of this preliminary study included the lack of a control group without sepsis and the length (up to 48 h) and variability of the time from onset of severe sepsis to sample acquisition. It is possible that some patients had higher biomarker levels earlier in the course of sepsis and these levels were decreasing at the time the samples were taken, which may account for the degree of heterogeneity demonstrated in the high-risk patients. However, determining the degree of heterogeneity in high-risk patients was not a primary goal of this investigation.

In our cohort of children with severe sepsis, we demonstrated that plasma biomarkers and cytokines can be measured accurately in real time using a novel microfluidic device. Such a platform allows these biomarkers to be used as the basis of enrichment strategies in critically ill patients. ■

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Erin F. Carlton, M.D.*
University of Michigan School of Medicine
Ann Arbor, Michigan

Walker M. McHugh, M.S.
University of Michigan School of Medicine
Ann Arbor, Michigan
and
PreDxion Bio, Inc.
South San Francisco, California

Kelli McDonough, M.S.
Julie Sturza, M.P.H.
Karl Desch, M.D.
Timothy T. Cornell, M.D.
University of Michigan School of Medicine
Ann Arbor, Michigan

ORCID ID: 0000-0003-3194-8833 (W.M.M.).

*Corresponding author (e-mail: ecarlton@med.umich.edu).

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Endobronchial Ultrasound–guided Biopsy Is Feasible, Safe, and Improves Diagnostic Yields in Immunocompromised Children

To the Editor:

Radial endobronchial ultrasound (R-EBUS) is widely used in adults for transbronchial biopsy (TBB) guidance and is predominantly performed for lung tumor diagnosis. The use of R-EBUS in children has not been described. Immunocompromised children represent a unique population of patients who may benefit from this technique. Pneumonia accounts for approximately 75% of all pulmonary complications in immunocompromised patients (1). Immunodeficiency may be a primary immunodeficiency or secondary to solid organ transplantation, hematopoietic stem cell transplantation, and immunosuppressive agents (2, 3). As a result of immunodeficiency, pulmonary complications in these patients can present with unusual features or subtle manifestations, creating diagnostic challenges. In addition, immunocompromised patients with pulmonary manifestations may be treated empirically with broad-spectrum antibiotics before BAL sampling, thus reducing diagnostic yield.

Early BAL and TBB with flexible bronchoscopy have been shown to reduce morbidity and mortality in immunocompromised adults with pneumonia (4). Sensitivity estimates for TBB under fluoroscopic guidance range between 34% and 63% depending on the size of the lesions (5). The addition of R-EBUS TBB has been established to improve sensitivity, specificity, and diagnostic accuracy in adults (6, 7). In January 2014, we introduced R-EBUS TBB into our clinical practice for immunocompromised children with opacities on chest imaging. Here, we provide the first report of the use of this diagnostic modality in a pediatric population and provide data on the feasibility, safety, and diagnostic value of R-EBUS TBB in immunocompromised children with pulmonary lesions.

Methods

Patients were selected by retrospective review under an institutional review board–approved protocol at the Children’s Hospital of Philadelphia. We included all immunocompromised children with pulmonary lesions who underwent R-EBUS TBB and BAL between January 2014 and July 2018. The electronic medical record data collected included age, height, weight, underlying diagnoses, immune status, chemotherapeutic agents used, the ventilation

method used during the procedure (laryngeal mask airway or endotracheal tube), bronchoscope sizes, therapies (antibacterial, antifungal, or antiviral) within the last 30 days before the procedure, and microbiologic results (staining, culture, and PCR based) obtained within 30 days before the procedure. The outcome data collected included postprocedure microbiologic therapy changes, related complications, days until discharge, and survival. All procedures were completed under general anesthesia with mechanical ventilatory support. Patients who were large enough to accommodate an endotracheal tube > 5.5 mm were intubated for the procedure. All procedures were performed using an Olympus electronic flexible bronchoscope with a 2-mm instrument channel (BF-MP160F, BF-P190, or BF-H190), a 2-mm standard guide sheath, a radial ultrasound probe, and endoscopic forceps (1.5 and/or 2.0 mm). Lesions were identified before the procedure by chest computed tomography scans in all patients. Attempts were made to visualize these lesions by inserting the radial ultrasound probe through the flexible bronchoscope into the corresponding affected lobes or segments, and lesions were sampled with endoscopic forceps. Fresh BAL and R-EBUS TBB samples in sterile saline were immediately transported to the microbiology laboratory. BAL cultures and R-EBUS TBB samples were processed for bacterial, fungal, viral, and acid-fast bacilli. BAL analyses of total cell counts with differential, silver staining, and cytology, and pathology of R-EBUS TBB specimens were performed in most cases. Organisms detected were determined to be “clinically significant” based on decisions made by the medical team and individual reviews of patient cases by the authors. For statistical analyses, demographic and clinical characteristics of the patients were evaluated by means of contingency tables with the chi-square Fisher’s exact test. BAL and BAL plus R-EBUS TBB microbiologic results were compared using the Wilcoxon matched-pairs signed-rank test. Days to discharge and mortality rates were evaluated by means of survival curve comparisons and the Mantel-Cox log-rank test.

Results

Eighteen patients (2.5–18 yr old) underwent a total of 19 R-EBUS procedures. Pulmonary nodules ranging from 6 to 28 mm in diameter were identified in 17 of the patients. The nodules were isolated to a single lobe in 10 of these patients and were found to be multilobar in the rest of them. The demographics and clinical characteristics of the patients are listed in Table 1. Abnormal lung echogenicity corresponding to chest computed tomography findings was identified by R-EBUS in 16 patients (84.2%) from whom a successful biopsy was obtained for pathologic and microbial analyses.

Between patients in whom clinically significant pathogens were detected ($n = 10$) and those with no clinically significant pathogens detected ($n = 9$), there were no significant differences in age, height, weight, underlying diagnosis, immune status, chemotherapeutic agents used, ventilation technique used, number of nodules on chest imaging, diffuse versus lobar opacification classification, BAL neutrophil percentage, or size of the bronchoscope used. Because of the small number of patients, a chi-square calculation based on the duration of preprocedure antibiotics (no antibiotics, duration > 1 wk, or duration < 1 wk) could not be performed, and thus the calculation for preprocedure antibiotics was performed based on two groupings (antibiotics before procedure vs. no antibiotics). This showed no significant differences in the clinically significant pathogens identified.

Author Contributions: All authors contributed to the conception or design of the study, and acquisition, analysis, or interpretation of data; drafted and critically revised the manuscript for important intellectual content; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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