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VALIDATION OF A GENE EXPRESSION-BASED SUBCLASSIFICATION STRATEGY FOR PEDIATRIC SEPTIC SHOCK

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Abstract

Objective—Septic shock heterogeneity has important implications for clinical trial implementation and patient management. We previously addressed this heterogeneity by identifying 3 putative subclasses of children with septic shock based exclusively on a 100-gene expression signature. Here we attempted to prospectively validate the existence of these gene expression-based subclasses in a validation cohort.

Design—Prospective observational study involving microarray-based bioinformatics.

Setting—Multiple pediatric intensive care units in the United States.

Patients—Separate derivation (n=98) and validation (n=82) cohorts of children with septic shock.

Interventions—None other than standard care.

Measurements and Main Results—Gene expression mosaics of the 100 class-defining genes were generated for 82 individual patients in the validation cohort. Using computer-based image analysis, patients were classified into 1 of 3 subclasses ("A", "B", or "C") based on color and pattern similarity relative to reference mosaics generated from the original derivation cohort. After subclassification, the clinical database was mined for phenotyping. Subclass A patients had higher illness severity relative to subclasses B and C, as measured by maximal organ failure, fewer ICU-free days, and a higher PRISM score. Patients in subclass A were characterized by repression of genes corresponding to adaptive immunity and glucocorticoid receptor signaling. Separate subclass assignments were conducted by 21 individual clinicians, using visual inspection. The consensus classification of the clinicians had modest agreement with the computer algorithm.

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Conclusions—We have validated the existence of subclasses of children with septic shock based on a biologically relevant, 100-gene expression signature. The subclasses have relevant clinical differences.

Keywords

microarray; gene expression; stratification; staging; septic shock; pediatrics

INTRODUCTION

Septic shock is a heterogeneous syndrome with variable physiological and biological manifestations across patient groups [1, 2]. A major challenge in critical care medicine is the development of clinically feasible stratification strategies to manage this heterogeneity for the design of more effective clinical trials and individualized patient management [3, 4]. We are addressing the challenge of septic shock heterogeneity by leveraging the discovery potential of whole-genome expression profiling [5].

Viewing septic shock as a syndrome implies the existence of septic shock subclasses predicated on distinct biological processes leading to distinct clinical phenotypes. We previously identified three putative subclasses of children with septic shock based exclusively on differential gene expression patterns representing the first 24 hours of admission to the pediatric intensive care unit (PICU) [6]. Further analyses of the subclasses revealed important phenotypic differences, thus suggesting that the gene expression-based subclasses are clinically relevant. In a subsequent cross validation study, we demonstrated that clinicians could reliably assign patients to the putative subclasses based on 100 class-defining genes depicted as visually intuitive gene expression mosaics [7].

We now seek to prospectively validate our subclassification strategy in a new cohort of children with septic shock. We test the hypothesis that the differential expression patterns of 100 class-defining genes, depicted using visually intuitive expression mosaics, can be used to allocate a validation cohort into septic shock subclasses having relevant clinical differences and biologically relevant differential gene expression.

METHODS

Patients and data collection

The study protocol was approved by the Institutional Review Boards of each institution (n = 11) and is identical for both the derivation and validation cohorts. Children ≤10 years of age admitted to the PICU and meeting pediatric-specific criteria for septic shock were eligible [8]. Controls were recruited from the ambulatory departments of participating institutions using published inclusion and exclusion criteria [9, 10]. The derivation cohort of 98 patients with septic shock and 32 normal controls was previously published [6, 7]. The validation cohort consists of 82 new patients with septic shock and 21 new controls (control median age 2.9 years (1.3–5.6); 11 males and 10 females). The microarray data for both cohorts have been deposited in the NCBI Gene Expression Omnibus (Accession numbers: GSE26440 and GSE26378).

After informed consent from parents or legal guardians, blood samples were obtained within 24 hours of initial presentation to the PICU with septic shock. Clinical and laboratory data were collected daily while in the PICU and stored using a web-based database. The following variables were tracked as indicators of illness severity: organ failure, PRISM score [11], PICU free days, and mortality. Organ failure was defined using pediatric-specific criteria and tracked up to the first 7 days of PICU admission [8]. The calculation of PICU

free days was based on the difference between a maximum PICU admission of 28 days and the actual PICU days. Patients who died during the 28 day study period and patients that remained in the PICU for ≥28 days were assigned zero PICU free days. Mortality was tracked for 28 days after enrollment.

RNA extraction and microarray hybridization

Total RNA was isolated from whole blood using the PaxGene™Blood RNA System (PreAnalytiX, Qiagen/Becton Dickson, Valencia, CA). Microarray hybridization was performed as previously described using the Human Genome U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) [6, 9, 10, 12–14]. CEL files were preprocessed using Robust Multiple-Array Average (RMA) normalization and GeneSpring GX 7.3 software (Agilent Technologies, Palo Alto, CA). All signal intensity-based data were used after RMA normalization [15]. All chips were normalized to the respective median values of controls.

Gene expression mosaics

In the aforementioned study (the derivation cohort), we used discovery-oriented expression and statistical gene filters, and unsupervised hierarchical clustering, to identify 3 putative subclasses of children with septic shock (subclasses “A”, “B”, and “C”) [6]. We subsequently refined the subclass-defining signature to 100 genes using a 2 stage approach. In stage 1 we used K-means clustering to identify coordinately regulated gene clusters corresponding to signaling pathways related to inflammation and immunity. In stage 2 we tested the genes identified in stage 1 for the ability to predict the putative subclass and extracted the top 100 class-predictor genes [6]. These 100 class-predictor genes form the basis of the current study (see Supplemental Table).

Expression mosaics representing the 100 class-defining genes were generated using the Gene Expression Dynamics Inspector (GEDI) [16, 17]. The signature graphical outputs of GEDI are expression mosaics that give microarray data a “face” that is intuitively recognizable via human pattern recognition. The algorithm for creating the mosaics is a self-organizing map. Additional technical details regarding GEDI can be found at: http://www.childrenshospital.org/research/ingber/GEDI/gedihome.htm.

Computer-based classification

The reference mosaics representing the 100 class-defining genes are published [7], and represent the average expression patterns of the individual patients within a given subclass of the derivation cohort. For each patient in the validation cohort, the respective expression data for the 100 class-defining genes were uploaded to GEDI and individual expression mosaics were generated for each patient. Validation cohort patients were then subclassified using a public image analysis platform (ImageJ, http://rsbweb.nih.gov/ij/). The reference and the individual patient mosaics from the validation group were loaded onto ImageJ. The absolute difference in RGB pixel to pixel intensity was calculated for each individual patient mosaic in the validation cohort, relative to each of the three reference mosaics. Final subclassification was based on the “least difference” between the individual patient mosaic and one of the three reference mosaics.

Clinician-based subclassification

We also conducted clinician-based subclassification as previously described [7]. Twenty-one pediatric intensivists participated as evaluators. Each evaluator was shown the 82 individual patient mosaics from the validation cohort, and asked to classify each patient as subclass “A”, “B”, or “C”, based on color and pattern similarity relative to the reference mosaics. The clinicians were not provided with any additional instructions and were blinded

to clinical data. Clinician responses were catalogued and subsequently used to arrive at a final consensus subclassification based on the "majority call" (i.e. ≥8 calls for a given class).

Clinical phenotyping and data analysis

After the patients in the validation cohort were subclassified, we mined the clinical database to assess clinical differences between the 3 subclasses. Ordinal and continuous clinical variables not normally distributed were analyzed via 3 group ANOVA on Ranks. Dichotomous clinical variables were analyzed using a 2 by 3 contingency table and Chi-square test (SigmaStat Software, Systat Software, Inc., San Jose, CA).

RESULTS

Baseline Data

The characteristics of the derivation and validation cohorts are provided in Table 1. The validation cohort had a higher proportion of males, a lower proportion of patients with immune suppression, and a lower proportion of patients with negative microbiological cultures, compared to the derivation cohort. All other variables in Table 1 were not significantly different between the two cohorts.

The reference mosaics for the subclassification protocol are shown in the upper panel of Figure 1. The reference mosaics depict the average expression patterns of the same 100 class-defining genes and represent the 3 previously published derivation cohort septic shock subclasses [6, 7]. Examples of individual patient mosaics in the validation cohort, representing the same 100 class-defining genes, are shown in the lower panel of Figure 1.

Computer-Based Subclassification

Table 2 provides the characteristics of the validation cohort subclasses based on the computer algorithm. Subclass A patients had higher illness severity than subclass B and C patients, as measured by maximum number of organ failures and PRISM scores. Subclass A patients also had higher illness severity than subclass B patients, as measured by fewer PICU free days. There was a trend toward a higher mortality odds ratio in subclass A patients, relative to subclass B and C patients. Subclass A patients were also younger, and had a lower peripheral total white blood cell count and neutrophil count, compared to subclass B patients. All other variables in Table 2 were not significantly different across the 3 septic shock subclasses in the validation cohort. These data demonstrate that computer-based image analysis can allocate patients into gene expression-based septic shock subclasses having relevant clinical differences.

Clinician-Based Subclassification

In this analysis the clinical evaluators conducted the subclassification as described in the *Methods*. For 31 of the validation cohort patients (38%), all 21 evaluators provided the same subclassification. The other potential majority calls (ranging from 8 to 20) were relatively evenly distributed (Figure 2). Four patients received < 11 majority calls (i.e. less than 50% of the evaluators agreed to the same classification). The individual expression mosaics of these 4 patients and their respective 28 day outcomes are provided in Figure 3.

The κ coefficient to measure inter-evaluator agreement among the 21 evaluators was 0.633 (0 = no agreement; 1 = perfect agreement). Sixty-five of the validation cohort patients (79%) were allocated to the same subclass by both the computer algorithm and the consensus classification generated by the clinicians (κ coefficient = 0.688).

Table 3 provides the characteristics of the validation cohort subclasses based on the consensus classification of the 21 clinicians. Subclass A patients had higher illness severity than subclass B and C patients, as measured by maximum number of organ failures and PRISM score. Subclass A patients were also younger, and had a lower peripheral total white blood cell count and neutrophil count, compared to subclass B patients. All other variables in Table 3 were not significantly different across the 3 septic shock subclasses in the validation cohort. These data validate that clinicians can allocate patients with septic shock into gene expression-based subclasses having relevant clinical differences.

Clinical characteristics of the combined cohorts

Combined with our previous report [6], we have allocated a total of 180 patients into expression-based subclasses. The major clinical characteristics of the respective subclasses are consistent when comparing the current validation cohort and the original derivation cohort [6]. Table 4 provides the major clinical differences between the 3 subclasses in this combined cohort. Subclass A patients have a significantly higher mortality rate and mortality odds ratio than patients in subclasses B and C. Subclass A patients also have a higher number of organ failures, a higher PRISM score, and fewer PICU free days than patients in subclasses B and C. Finally, subclass B patients are older and have a higher proportion of females, compared to subclasses A and C.

Addressing potential confounding variables

Since we used whole blood-derived RNA it is possible that the class-defining gene expression patterns reflect differential white blood cell counts. Accordingly, we analyzed our expression data for the presence of previously published signature probe sets for neutrophils, lymphocytes, and monocytes, respectively [18]. Signature probe “presence” was defined using the following criteria: ≥100 raw expression value in a least one-half of the subjects in each subclass. Table 5 provides the results from this analysis and demonstrates that the signature probe sets were equally present across the 3 subclasses. Thus, the relative contributions of the three major leukocyte subsets, to the whole blood transcriptomic response, were relatively similar across the 3 subclasses. Accordingly, the differences in gene expression between the 3 subclasses do not appear to be artifacts of differential peripheral white blood cell counts.

The validation cohort had a significantly lower proportion of patients with negative cultures compared to the derivation cohort. Accordingly, we conducted a 2 group ANOVA (Benjamini-Hochberg False Discovery Rate = 5%) to determine if the 100 class-defining genes were differentially expressed between patients with negative cultures (n = 25) and patients with positive cultures (n = 57) in the validation cohort. This analysis revealed that none of the 100 subclass-defining genes were differentially expressed between the two groups, thus suggesting that microbiology culture status was unlikely to be a strong confounder.

The validation cohort also had a higher proportion of males compared to the derivation cohort. Accordingly, we conducted a 2 group ANOVA (Benjamini-Hochberg False Discovery Rate = 5%) to determine if the 100 class-defining genes were differentially expressed between males (n = 57) and females (n = 25) in the validation cohort. This analysis revealed that none of the 100 subclass-defining genes were differentially expressed between males and females, thus suggesting that gender was unlikely to be a strong confounder.

Since patients were recruited from multiple centers, it is possible that center-specific effects could confound the data. Table 6 provides the number of validation cohort patients in each

gene expression-based subclass based on enrollment site. Thirty seven patients (45%) in the validation cohort were recruited from Site 1. Also, site 1 contributed a smaller proportion of subclass A patients, compared to subclass C patients. There were no other significant differences regarding enrollment among the other sites. Thus, we cannot rule out the possibility that the data are confounded, in part, by center-specific effects.

DISCUSSION

Tang et al. recently conducted a systematic review of microarray-based expression profiling studies in human sepsis [19]. One conclusion from this review is that the transcriptomic response is highly variable in human sepsis. Our previous data are consistent with this conclusion by demonstrating the potential existence of 3 subclasses of children with septic shock, as defined by variable gene expression. We have now prospectively validated that a 100-gene expression signature, depicted using visually intuitive mosaics, can be used to allocate patients with septic shock into subclasses having clinically relevant phenotypic differences and biologically relevant differential gene expression. While the subclassification strategy needs to be further refined, and one would expect that computer-based subclassification would be superior to that of clinician-based subclassification, we have nonetheless demonstrated that clinically relevant subclasses of children with septic shock can be identified via gene expression profiling.

The assertion that the subclasses have clinically relevant phenotypes is based on a higher level of illness severity in the subclass A patients, as measured by mortality, maximal number of organ failures, PRISM scores, and PICU free days. The profound negative impact of multiple organ failure on outcomes in critical illness is well established [20-22], and is therefore a clinically relevant measure. The reliance on PRISM scores could lead one to conclude that it would be more straightforward to calculate PRISM scores as a means of stratification. However, illness severity scores such as PRISM and APACHE are intended for population-based predictions, rather than for individual patient stratification, and do not provide biological information [23].

Patients in validation cohort subclass A had a trend toward higher mortality, which did not reach statistical significance. However, when we combined both the derivation and validation cohorts, the subclass A patients have a significantly higher mortality rate. Notably, the mortality rates of the patients in subclasses B and C are consistent with current estimates for the U.S. [24–26], whereas the mortality rate in subclass A patients is 3-fold higher.

The assertion that the subclasses have biologically relevant differences in gene expression is based on the functional significance of the 100 class-defining genes, which are enriched for genes corresponding to adaptive immunity [6, 27]. The majority of these genes corresponding to adaptive immunity are repressed in subclass A patients, relative to subclass B and C patients [6]. It is unlikely that lymphopenia accounts for this observation because the absolute lymphocyte counts were not significantly different between the 3 subclasses. Recent literature indicates that enhancement of adaptive immune function may be a rational therapeutic strategy in sepsis [28–33]. Optimization of such a strategy, however, will need to take into account the potential existence of subclass A patients, characterized by a higher illness severity and repression of adaptive immunity-related genes.

The 100 class-defining genes are also enriched for genes corresponding to glucocorticoid receptor signaling [6, 27], and these genes are also repressed in subclass A patients, relative to subclass B and C patients [6]. Glucocorticoid replacement therapy and the concept of relative adrenal insufficiency in septic shock are highly controversial topics in critical care

medicine [34–36]. The potential existence of a subclass of patients with septic shock (i.e. subclass A) having a higher illness severity and repression of genes corresponding glucocorticoid receptor signaling, may have important implications for future clinical trials and may have been confounders in previous clinical trials.

Subclass A patients were significantly younger than subclass B patients in both our previous study [6] and the current study. This observation is consistent with pediatric septic shock epidemiology, which has identified young age as a risk factor for increased illness severity [24–26]. However, subclass C patients are of a similar age to subclass A patients, but have a lower illness severity in both studies. Thus, while younger age is a risk factor for illness severity, the current data indicate that the expression pattern of the 100 class-defining genes also impacts illness severity, independent of age.

Our subclassification strategy is focused on a single time point and therefore does not take into account potential temporal shifts in patient status from one subclass to another. However, the major goal of the strategy is to allocate patients into subclasses at a time point that affords earlier clinical management decisions or early stratification for clinical trials [4, 37, 38].

In conclusion, we have addressed the challenges of septic shock heterogeneity and stratification by prospectively validating the existence of septic shock subclasses based on a 100-gene expression signature. The subclasses can be identified at a clinically relevant time point and have relevant clinical differences. Finally, the expression patterns of the 100 class defining-genes may therapeutic implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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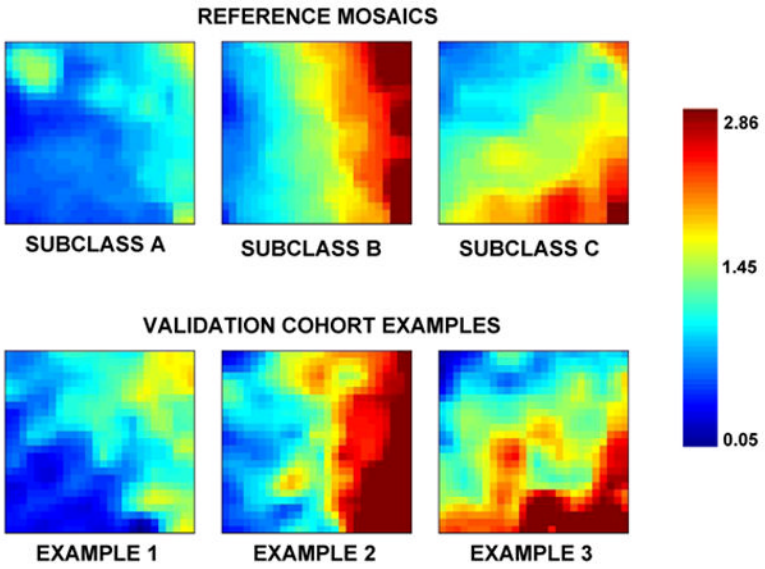

Figure 1.

GEDI-generated reference mosaics (top panel) and examples of GEDI-generated individual patient mosaics from the validation cohort (bottom panel). The reference mosaics are derived from the previously published derivation cohort (Refs. 6 and 7) and represent the mean expression values for patients in the respective subclasses. Both the reference mosaics and the individual validation cohort examples depict the expression levels of the same 100 class-defining genes. The color bar on the right provides the relative gene expression based on the respective color intensities.

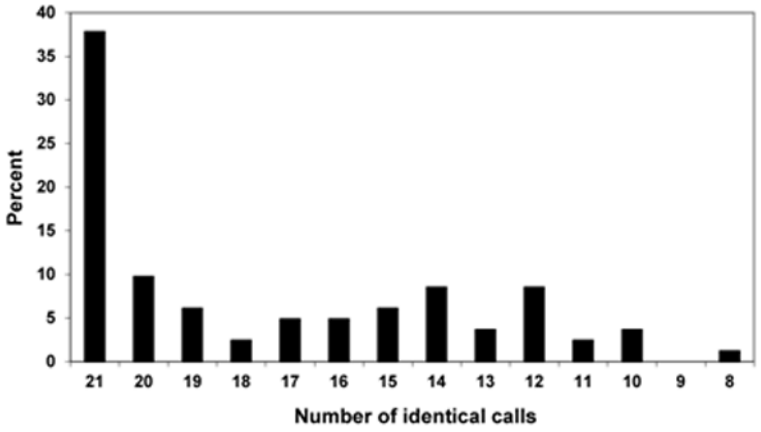

Figure 2.
The distribution of potential majority calls (maximum = 21; minimum = 8) among the 21 clinical evaluators. These majority calls led to the final consensus classification for the 82 patients in the validation cohort.

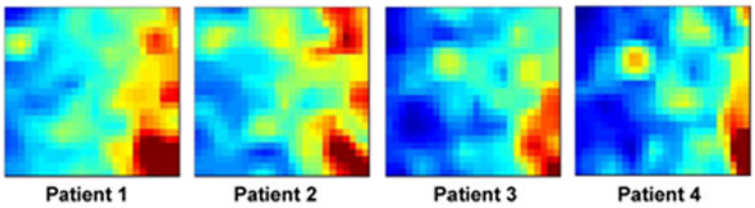

Figure 3.

The individual expression mosaics of the four patients in the validation cohort that received < 11 majority calls by the clinical evaluators. Patient 1 (non-survivor) and Patient 2 (survivor) were allocated to subclass C by the computer algorithm. Patient 3 (non-survivor) and Patient 4 (survivor) were allocated to subclass A by the computer algorithm.

Table 1

Demographics and clinical characteristics of the derivation and validation cohorts.[1]

	Derivation Cohort	Validation Cohort	P value
No. of patients	98	82	--
Median age in years (IQR)[2]	2.2 (1.0–5.0)	2.4 (0.8–6.5)	0.860
No. of males/females	52/46	57/25	0.036
No. of deaths (%)	18 (18)	12 (15)	0.639
Maximum # of organ failures (IQR) [3]	2 (2–3)	2 (2–3)	0.859
Median PRISM score (IQR)	16 (11–21)	14 (9–19)	0.081
No. with co-morbidity (%)[4]	41 (42)	33 (40)	0.949
No. with immune suppression (%)[5]	23 (23)	7 (8)	0.013
No. receiving hydrocortisone (%)[6]	35 (36)	34 (42)	0.525
No. with gram pos. bacteria (%)[7]	23 (23)	24 (29)	0.477
No. with gram neg. bacteria (%)	20 (20)	26 (32)	0.119
No. with negative cultures (%)	45 (46)	25 (30)	0.050
Median WBC count × 10^3/mm^3 (IQR)	13 (4–20)	14 (7–18)	0.446
Median neutrophil count × 10^3/mm^3 (IQR)	7 (3–13)	10 (4–16)	0.110
Median lymphocyte count × 10^3/mm^3 (IQR)	1.7 (0.8–3.1)	2.1 (0.9–3.8)	0.605
Median monocyte count × 10^3/mm^3 (IQR)	0.5 (0.2–1.3)	0.5 (0.3–1.0)	0.878

[1] Continuous variables are analyzed by ANOVA on Ranks, and proportions are analyzed by Chi-square.

[2] Interquartile range (IQR).

[3] Refers to the maximum number of organ failures during the initial 7 days of PICU admission.

[4] Refers to patients having any major diagnosis in addition to septic shock (e.g. trauma, sickle cell disease, congenital heart disease, liver failure, etc.)

[5] Refers to patients with immune deficiency secondary to an intrinsic documented defect of the immune system, or patients receiving immune-suppressive medications (e.g. calcineurin inhibitors or high dose corticosteroids).

[6] For cardiovascular shock.

[7] All bacterial culture data refer to samples obtained from bodily fluids that are normally sterile (i.e. blood, urine, cerebral spinal fluid, broncho-alveolar lavage, and / or peritoneal fluid).

Table 2

Demographics and clinical data for the validation cohort septic shock subclasses using computer-based image analysis.[1]

	Subclass A	Subclass B	Subclass C	P value
No. of patients	27	24	31	--
Median age in years (IQR) [2]	1.1 (0.3–3.4)	6.0 (1.6–8.6)	2.4 (1.2–5.3)	0.006
No. of males/females	20/7	14/10	23/8	0.933
No. of deaths (%)	6 (22)[3]	2 (8)	4 (13)	0.353
Maximum # of organ failures (IQR) [4]	3 (2–4)	2 (2–3)	2 (2–3)	0.001
Median PRISM score (IQR)	17 (11–27)	10 (7–12)	14 (8–18)	0.003
Median PICU free days (IQR)	14 (0–20)	21 (18–23)	17 (7–24)	0.048
No. with co-morbidity (%)[5]	8 (30)	12 (50)	13 (42)	0.324
No. with immune suppression (%)[6]	2 (7)	2 (8)	3 (10)	0.953
No. receiving hydrocortisone (%)[7]	10 (37)	13 (54)	11 (35)	0.321
No. with gram pos. bacteria (%)[8]	9 (33)	7 (29)	8 (26)	0.821
No. with gram neg. bacteria (%)	11 (41)	6 (25)	9 (29)	0.445
No. with negative cultures (%)	5 (18)	8 (33)	12 (39)	0.234
Median WBC count × 10^3/mm^3 (IQR)	9 (4–14)	15 (11–24)	16 (8–18)	0.009
Median neutrophil count × 10^3/mm^3 (IQR)	7 (3–11)	14 (10–22)	10 (4–16)	0.010
Median lymphocyte count × 10^3/mm^3 (IQR)	1.8 (0.9–4.3)	2.2 (0.9–3.2)	2.1 (1.0–3.9)	0.857
Median monocyte count × 10^3/mm^3 (IQR)	0.5 (0.3–0.8)	0.7 (0.3–1.0)	0.5 (0.2–1.6)	0.899

[1] Continuous variables are analyzed as 3 group comparisons using ANOVA on Ranks and 2 degrees of freedom. Proportions are analyzed as 3 group comparisons using a 2 by 3 contingency table and Chi-square with 2 degrees of freedom.

[2] Interquartile range (IQR).

[3] Odds ratio for mortality vs. Subclass B: 3.1 (0.6–17.3); vs. Subclass C: 1.9 (0.5–7.7).

[4] Refers to the maximum number of organ failures during the initial 7 days of PICU admission.

[5] Refers to patients having any major diagnosis in addition to septic shock (e.g. trauma, sickle cell disease, congenital heart disease, liver failure, etc.)

[6] Refers to patients with immune deficiency secondary to an intrinsic documented defect of the immune system, or patients receiving immune-suppressive medications (e.g. calcineurin inhibitors or high dose steroids).

[7] For cardiovascular shock.

[8] All bacterial culture data refer to samples obtained from bodily fluids that are normally sterile (i.e. blood, urine, cerebral spinal fluid, broncho-alveolar lavage, and/or peritoneal fluid).

Table 3

Demographics and clinical data for the validation cohort septic shock subclasses based on the majority call of 21 clinical evaluators.[1]

	Subclass A	Subclass B	Subclass C	P value
No. of patients	25	28	29	--
Median age in years (IQR)[2]	1.0 (0.2–3.1)	5.7 (1.0–8.6)	2.0 (1.3–5.4)	0.005
No. of males/females	17/8	19/9	21/8	0.915
No. of deaths (%)	5 (20)[3]	4 (14)	3 (10)	0.520
Maximum # of organ failures (IQR) [4]	3 (2–4)	2 (2–3)	2 (2–3)	0.032
Median PRISM score (IQR)	17 (11–28)	11 (7–14)	14 (9–17)	0.014
Median PICU free days (IQR)	15 (1–20)	21 (13–23)	17 (9–24)	0.191
No. with co-morbidity (%)[5]	7 (28)	16 (57)	10 (34)	0.071
No. with immune suppression (%)[6]	1 (4)	3 (11)	3 (10)	0.627
No. receiving hydrocortisone (%)[7]	7 (28)	16 (57)	11 (36)	0.088
No. with gram pos. bacteria (%)[8]	8 (32)	7 (25)	9 (31)	0.827
No. with gram neg. bacteria (%)	8 (32)	8 (29)	10 (34)	0.891
No. with negative cultures (%)	6 (24)	10 (36)	9 (31)	0.650
Median WBC count × 10^3/mm^3 (IQR)	10 (4–14)	16 (11–24)	15 (6–18)	0.009
Median neutrophil count × 10^3/mm^3 (IQR)	7 (1–11)	12 (8–21)	10 (4–16)	0.006
Median lymphocyte count × 10^3/mm^3 (IQR)	1.8 (1.0–4.1)	2.4 (0.9–3.5)	1.6 (0.9–3.8)	0.945
Median monocyte count × 10^3/mm^3 (IQR)	0.5 (0.4–1.0)	0.7 (0.3–1.0)	0.5 (0.2–1.2)	0.952

[1] Continuous variables are analyzed as 3 group comparisons using ANOVA on Ranks and 2 degrees of freedom. Proportions are analyzed as 3 group comparisons using a 2 by 3 contingency table and Chi-square with 2 degrees of freedom.

[2] Interquartile range (IQR).

[3] Odds ratio for mortality vs. Subclass B: 1.5 (0.4–4.6); vs. Subclass C: 2.2 (0.5–10.2).

[4] Refers to the maximum number of organ failures during the initial 7 days of PICU admission.

[5] Refers to patients having any major diagnosis in addition to septic shock (e.g. trauma, sickle cell disease, congenital heart disease, liver failure, etc.)

[6] Refers to patients with immune deficiency secondary to an intrinsic documented defect of the immune system, or patients receiving immune-suppressive medications (e.g. calcineurin inhibitors or high dose steroids).

[7] For cardiovascular shock.

[8] All bacterial culture data refer to samples obtained from bodily fluids that are normally sterile (i.e. blood, urine, cerebral spinal fluid, broncho-alveolar lavage, and/or peritoneal fluid).

Table 4

Major clinical characteristics of combined derivation and validation cohorts.[1]

	Subclass A	Subclass B	Subclass C	p value
No. of patients	55	69	56	--
Median age in years (IQR)[2]	0.7 (0.3–2.7)	5.2 (1.9–7.3)	2.2 (1.2–2.7)	<0.001
No. of males/females	39/16	33/36	37/19	0.020
No. of deaths (%)	16 (29)[3]	7 (10)	7 (12)	0.012
Max. # of organ failures (IQR)	3 (2–4)	2 (2–3)	2 (2–2)	<0.001
Median PRISM score (IQR)	19 (13–27)	11 (10–15)	12 (9–16)	<0.001
Median PICU free days (IQR)	11 (0–18)	22 (17–24)	19 (16–24)	0.002

[1] Continuous variables are analyzed as 3 group comparisons using ANOVA on Ranks and 2 degrees of freedom. Proportions are analyzed as 3 group comparisons using a 2 by 3 contingency table and Chi-square with 2 degrees of freedom.

[2] Interquartile range (IQR).

[3] Odds ratio for mortality vs. Subclass B: 3.6 (1.4– 9.6), p = 0.007; vs. Subclass C: 2.9 (1.1–7.7), p = 0.03.

Table 5

Presence of leukocyte subset signature probes across the three gene expression-based subclasses. Data presented as number of signature probes present (see text for presence criteria).

	Neutrophil probes (n = 38)	Lymphocyte probes (n = 50)	Monocyte probes (n = 28)
Subclass A	33	43	20
Subclass B	33	43	21
Subclass C	33	44	20

Table 6

Number of patients in each gene expression-based subclass based on enrollment site.

	Subclass A	Subclass B	Subclass C	Total
Site 1	8[1]	11	18	37
Site 2	2	1	1	4
Site 3	1	1	4	6
Site 4	1	3	0	4
Site 5	4	4	2	10
Site 6	3	0	1	4
Site 7	1	1	2	4
Site 8	1	0	0	1
Site 9	3	0	1	4
Site 10	2	1	1	4
Site 11	1	2	1	4

[1] $p < 0.05$ vs. subclass C (Chi-square with 2 degrees of freedom).