Host gene expression signatures to identify infection type and organ dysfunction in children evaluated for sepsis: a multicenter cohort study

Prof. Luregn J Schlapbach[1,2,3*#], PhD, Devika Ganesamoorthy[1*], PhD, Clare Wilson[4*], BChir, Sainath Raman[1,3], MBBS, PhD, Shane George[1,5,6], MPH, Peter J. Snelling[5,6], MPHTM, Natalie Phillips[1,7], MPhil, Adam Irwin[8,9], PhD, Natalie Sharp[1,3], BSc, Renate Le Marsney[1] MPH, Arjun Chavan[10], MD, Allison Hempenstall[11], MPH, Seweryn Bialasiewicz[12], PhD, Anna D. MacDonald[1], PhD, Prof. Keith Grimwood[6,13], MD, Jessica Kling[14], PhD, Stephen McPherson[14], PhD, Antje Blumenthal[14], Dr rer. nat, Myrsini Kaforou[4], PhD, Prof. Michael Levin[4], PhD, Jethro A Herberg[4], PhD, Kristen S Gibbons[1], PhD, Lachlan J.M. Coin[15,16*], PhD, for the EUCLIDS consortium and the RAPIDS Study Group

[*]contributed equally

[#]corresponding author

[1] Children's Intensive Care Research Program (ChIRP), Child Health Research Centre, Faculty of Medicine, The University of Queensland, Brisbane, Queensland, Australia

[2] Department of Intensive Care and Neonatology, and Children's Research Center, University Children's Hospital Zurich, University of Zurich, Zurich, Switzerland

[3] Paediatric Intensive Care Unit, Queensland Children's Hospital, Children's Health Queensland, Brisbane, Queensland, Australia

[4] Section of Paediatric Infectious Disease, Faculty of Medicine, Imperial College London, London, United Kingdom

[5] Department of Emergency Medicine, Gold Coast University Hospital, Southport, Queensland, Australia

[6] School of Medicine and Dentistry and the Menzies Health Institute Queensland, Griffith University, Southport, Queensland, Australia

[7] Emergency Department, Queensland Children`s Hospital, Childrens Health Queensland, Brisbane, Queensland, Australia

[8] Infection Management and Prevention Services, Queensland Children's Hospital, Children's Health Queensland, Brisbane, Queensland, Australia

[9] UQ Centre for Clinical Research, The University of Queensland, Brisbane, Queensland, Australia

[10] Paediatric Intensive Care Unit, Townsville University Hospital, Townsville, Queensland, Australia

[11] Thursday Island Base Hospital, Thursday Island, Queensland, Australia

[12] School of Chemistry and Molecular Biosciences, The Australian Centre for Ecogenomics, The University of Queensland, Brisbane, Queensland, Australia

[13] Department of Infectious Disease and Paediatrics, Gold Coast Health, Southport, Queensland, Australia

[14] Frazer Institute, Faculty of Medicine, The University of Queensland, Brisbane, Queensland, Australia

[15] Doherty Institute, University of Melbourne, Melbourne, Victoria, Australia

[16] Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland, Australia

Correspondence

Prof. Luregn Schlapbach, MD, PhD

Head, Pediatric and Neonatal Intensive Care Unit

University Children's Hospital Zurich – Eleonore Foundation

Steinwiesstrasse 75

CH-8032 Zurich Switzerland

Phone +41 44 266 71 11

Email: luregn.schlapbach@kispi.uzh.ch

Word count manuscript: 3453

Figures: 2

Tables: 4

Research in context

Evidence before this study

Sepsis remains one of the leading causes of childhood mortality and morbidity globally. While sepsis is defined as dysregulated host response to infection leading to organ dysfunction, criteria for sepsis remain based on physiological measures of organ dysfunction rather than biological markers of host response. The risk of overusing antimicrobials lends further urgency to develop and validate novel pathogen group-specific sepsis markers. There is thus an unmet need for point of care tests identifying host response specific to bacterial versus viral infection leading to organ dysfunction in children. Whole blood human transcriptomic analyses have emerged as a promising approach to characterize the host response, but most previous studies on septic patients included adults, were limited to differentiating bacterial versus viral infection, or focussed on mortality as an outcome in ICU patients. Furthermore, the majority of previous studies used multi-array rather than RNAseq, and the size of included cohorts was relatively small. We searched publications in English language in PubMed since January 2011 with the terms “child OR paediatric”, “sepsis OR septic shock”, “infection”, “bacterial”, “viral” AND “transcriptomics OR multiarray OR RNAseq” to identify relevant previous studies.

Added value of this study

In this large cohort investigating host gene expression signatures to identify sepsis in children, patients were recruited through a multicenter prospective Australian study, and split into a discovery (n=595) and a validation (n=312) cohort. An external validation cohort consisted of children with infection (n=362) recruited through a European consortium. We derived and validated two novel gene expression signatures – a 10-gene signature to discriminate bacterial from viral infection, and a 10-gene signature to identify the development of organ dysfunction. This novel sepsis signatures achieved an AUC of 90.5% (95%-CI 83.3% - 97.6%) and 94.7% (95%-CI 87.8% - 100.0%) in the validation dataset at identifying organ dysfunction within 24 hours specific for bacterial, and viral infection, respectively.

Implications of all the available evidence

A novel host gene expression signature can identify type of infection and organ dysfunction in children evaluated for sepsis. Findings were robust across a number of severity outcomes, such as need for organ support, need for vasopressors, multi-organ failure, and organ failure remote from the organ of infection. In addition, we compared the performance of this novel transcriptomic signature to previously published infection-specific, or severity-specific signatures, providing for the first time independent validation of previously reported signatures. Whether the implementation of such signatures into point-of-care tests provides actionable information on treatable traits at the bedside, potentially leading to improved use of antibiotics, needs to be tested in future trials.

Abstract

Background: Sepsis is defined as dysregulated host response to infection leading to life-threatening organ dysfunction. Biomarkers characterising dysregulated host response in sepsis are lacking. We aimed to develop host gene expression signatures predicting organ dysfunction in children with bacterial versus viral infection.

Methods: Prospective observational study in four Emergency Departments and Intensive Care Units in Australia. 907 children aged 1 month to 17 years evaluated for sepsis were recruited between September 2017 and October 2021. Patients were split into discovery (n=595) and validation (n=312) cohorts. An external validation cohort consisted of 362 children with infection. Whole blood RNA sequencing was performed using Illumina NovaSeq. Feature selection approaches were applied to discover novel gene signatures for infection type and organ dysfunction. The primary endpoint was the presence of organ dysfunction at 24 hours of sampling in the presence of confirmed bacterial, versus viral infection.

Findings: We identified a novel 10-gene disease-class signature, which achieved an Area under the Curve (AUC) of 94.1% (95%-confidence interval [CI], 90.6% - 97.7%) to discriminate bacterial from viral infection in the validation cohort. We also identified a novel 10-gene disease-severity signature to predict the development of organ dysfunction within 24 hours with an AUC of 82.2% (95%-CI 76.3% - 88.1%) in the validation cohort. In combination, the disease-class and disease-severity signatures achieved an AUC of 90.5% (95%-CI 83.3% - 97.6%) and 94.7% (95%-CI 87.8% - 100.0%) at predicting organ dysfunction within 24 hours of sampling in predicted bacterial, and viral infection, respectively. In the external dataset, the signatures achieved an AUC of 90.9% (95%-CI 85.0% - 96.9%) for bacterial/viral classification and an AUC of 70.1% (95%-CI 44.1% - 96.2%) for identifying organ dysfunction at time of sampling in patients with predicted bacterial infection.

Interpretation: In children evaluated for sepsis, novel host transcriptomic signatures specific for bacterial and viral infection can identify dysregulated host response leading to organ dysfunction.

Funding: Medical Research Future Fund and several additional grants.

Introduction

Sepsis is defined as a dysregulated host response to infection leading to life-threatening organ dysfunction[1]. Sepsis remains a leading cause of mortality in paediatric age groups with over 3 million annual deaths attributable to sepsis[2]. In the United States alone, paediatric sepsis was estimated to account for $7.31 billion direct costs in 2016[3], and one in five survivors will develop new or progressive medical conditions after sepsis[4]. However, most paediatric infections are viral resulting in particular challenges in recognizing sepsis in this age group. Therefore, campaigns providing incentives for early administration of antimicrobials have been criticized as they may potentially encourage unnecessary use of antibiotics[5]. Despite progress in microbiological diagnostics, their turnaround time and accuracy remains inadequate to guide initial empiric treatment and lacks the ability to predict disease severity. Rapid diagnostics have enormous potential to enhance timeliness and accuracy of sepsis treatment, as well as of reducing inadvertent antibiotic usage.

To date, the mechanisms underpinning dysregulated host response(s) characterizing the progression from uncomplicated infection towards infection with organ dysfunction remain poorly elucidated[6-8]. There is an unmet need for diagnostic markers characterizing the progression of simple infection to one with organ dysfunction, specific for the type of infection. The ideal sepsis biomarker would yield information on the presence and type of the underlying infection (to guide decisions on antibiotics), as well as the likelihood of developing organ dysfunction (to guide decisions on treatment escalation and resuscitation, such as fluids, inotropes, and intensive care unit [ICU] admission), and be translatable into a point-of-care platform. Recently, host transcriptomic biomarkers have shown great promise in differentiating between viral and bacterial infections[9,10].

We hypothesized that RNA sequencing of whole blood would identify distinct host response patterns characteristic for viral versus bacterial infection, and that these can be combined with specific patterns characterizing children developing infection-associated organ dysfunction versus those without organ dysfunction. Accordingly, we designed the Rapid Paediatric Infection Diagnosis in Sepsis (RAPIDS) multi-center prospective cohort, to develop and validate markers of the early host response in children evaluated for sepsis.

Methods

Study design and oversight

This prospective multi-center cohort was performed at four hospitals in Queensland, Australia (**eMethods 1)**. The study reporting follows the Standards of Reporting of Diagnostic Accuracy Studies 2015 Update[11]. The institutional Human Research Ethics Committee approved the study (HREC/17/QRCH/85, June 9th 2017; **eMethods 2**). Written informed consent or delayed consent was obtained for all participants from their parents/carers (**eMethods 3**).

Patients

Children aged over 1 month to 17 years evaluated for sepsis at the participating Emergency Departments (ED) and ICUs were eligible if they underwent a diagnostic work-up for suspected sepsis, including blood cultures upon admission (**eMethods 3)**.

Study procedures

We obtained 2.5mL blood in PAXgene RNA tubes (PreAnalytix GMBH, Switzerland) simultaneously with routine clinical testing including blood cultures, blood counts, C-reactive protein, and microbiological investigations such as nasopharyngeal swabs as indicated clinically. A REDCap study database[12] prospectively captured information on demographics,

symptoms, comorbidities, microbiology results, antimicrobial treatment, and severity. Disease severity was assessed at baseline (time of blood sampling) and at 24 hours using clinical, laboratory, and organ support criteria for organ dysfunction defined by the 2005 International Pediatric Sepsis Definition Consensus Conference[13,14]. Accordingly, presence of organ dysfunction (cardiovascular, respiratory, neurologic, renal, hepatic, haematologic) was adjudicated. The infection status was categorized into definite bacterial (DB), definite viral (DV), probable bacterial (PB), probable viral (PV), combined bacterial and viral (CBV), non-infectious (NI) and unknown based upon a previously validated approach[9] (**eFigure 1**; **eMethods 4**). Two assessors experienced in paediatric critical care and infectious diseases independently verified the infection status using clinical records, microbiologic results, laboratory data and discharge reports. Adjudication of the final clinical phenotype required agreement of both assessors; in case of disagreement, a third senior assessor reviewed cases with the two assessors to ensure robust adjudication of clinical phenotypes (**eMethods 5**).

Endpoints

The primary outcomes were the presence of organ dysfunction at 24 hours of sampling in children with DB infection, and in children with DV infection. This outcome was constructed by combining the infection phenotype category (restricted to DB, DV, PB, PV, NI), with the adjudication by organ dysfunction at 24 hours (i.e. presence of any organ dysfunction versus no organ dysfunction at 24 hours). Given the lack of a gold standard for sepsis severity[15], several secondary severity outcomes were defined: (i) organ dysfunction remote from the primary focus of infection (as a proxy of organ dysfunction caused by a systemic process related to infection[16]); (ii) need for organ support (invasive or non-invasive respiratory support, inotropes/vasopressors, renal replacement, extracorporeal membrane oxygenation); (iii) need for inotrope/vasopressors; (iv) multi-organ dysfunction; (v) presence of cardiovascular,

respiratory, or neurologic dysfunction[15,17]; and (vi) type of organ dysfunction. These outcomes were assessed at 24 hours from sampling, as well as at time of sampling; with an additional secondary severity outcome created by the dynamics within the first 24 hours (worsening or improving) (**eFigure 2**).

RNA sequencing for discovery and validation cohort

Samples were stored at -80^{o}C until extraction. RNA was purified from samples using PAXgene Blood miRNA kits (PreAnalytix). Library preparation and sequencing were conducted at Institute for Molecular Biosciences Sequencing Facility (University of Queensland, Australia). The TruSeq RNA Ribo Zero Kit (Illumina) was used for ribosomal RNA depletion and sequencing library preparation. Libraries were sequenced on a NovaSeq Sequencer (Illumina) to generate at least 20 million sequencing reads per sample. The RNA sequencing configuration was 75bp single-end (50 samples), 100bp single-end (545 samples) and 100bp paired-end (316 samples), respectively. FastQC[18] and MultiQC[19] were used to assess the quality of sequencing reads. The first two batches of samples were used for discovery (n=595) and the third batch was used for validation (n=316). For the discovery cohort, the sample size was based on power to detect differential gene expression between conditions with 1.2-fold change, assuming 20 million reads per sample. According to RNASeqPower package in R, we required at least 78 samples per condition to achieve 80% power. This was achieved for the majority of comparisons, including organ dysfunction, and definite bacterial vs definite viral. For the validation cohort, we used the methodology described in Burderer et al[20] to estimate that a sample size of 315 would allow us to correctly estimate the sensitivity and specificity of the test within +/-0.05 at 95% confidence. Samples with completed phenotyping, monitoring, and RNA extraction by March 2020 were included in the discovery cohort, the rest of the samples which were recruited by October 2021 composed the validation cohort. Four samples in the

validation cohort failed quality assessment and were excluded from analysis, leaving 595 samples in the discovery cohort, and 312 samples in the validation cohort.

Sequencing reads were mapped to the human reference genome (version hg38) using STAR aligner (version 2.7.6a)[21]. GENCODE version 35 gene transcript annotation was used for the alignment. HTSeq count (version 0.13.5)[22] was used to ascertain the number of reads mapped per gene. Principal component analysis (PCA) was performed to identify any outliers (**eFigure 3; eMethods 6**).

Differential Expression Analysis

DESeq2[23] was used for differential expression analysis between different phenotypes (bacterial versus viral; with versus without organ dysfunction). Genes with <10 read counts were excluded from analyses. Genes which had absolute log2 fold-change (LFC) of >1 and adjusted p-value of <0.05 were considered as differentially expressed (**eMethods 6**).

External Validation Cohort

RNA sequencing gene expression count data were obtained from the European Childhood Life-threatening Infectious Disease Study (EUCLIDS[24,25], **eMethods 1**, n=362). This observational study recruited children with severe infection in nine European countries between 2012–2016. Patients were phenotyped based on the likelihood of bacterial or viral infection[26] and considering severity at time of sampling.

Signature Discovery and Evaluation with FSPLS

Forward Selection Partial Least Squares (FSPLS, **eMethods 6**) was used to discover novel gene signatures to first distinguish infection types and to then predict presence of organ

dysfunction. The FSPLS approach enables simultaneous multiple comparisons to identify signatures which can be utilised to distinguish multiple phenotypes.

For disease-class signature analysis, FSPLS was run with five different comparisons (DB versus DV; DB versus PV; DV versus PB; DB versus NI; DV versus NI). Combined infections and unknown infections were not included in signature discovery. For severity signature analysis, FSPLS was run with those with versus those without organ dysfunction at 24-hours post sampling, and with those with versus those without organ dysfunction at the time of sampling. Disease-class stratified severity weights were obtained by running FSPLS on datasets stratified by predicted disease-class (viral, bacterial or non-infectious). To predict sepsis, firstly we used the novel disease-class signature to predict the infection types as either DB or DV or NI, as these groups have well-defined phenotypes (**eFigure 1; eMethods 6)**. Then, we applied the novel disease-severity signature for each infection type to identify organ dysfunction (**Figure 1**).

In order to benchmark the novel signatures, we used our dataset to refit previously published gene-expression signatures reported in patients with infection and sepsis (**eMethods 6 and 7**), specifically Herberg et al[9]**,** McHugh et al[10], Tang et al[27], Wong et al[28], Sweeney et al[29], Sampson et al[30], Li et al[31], Li et al[32] for disease-class and Lukaszewski et al[33], Pena et al[34], Irwin et al[35] and Baghela et al[36] for disease-severity. As the weights of the genes in the signatures were not publicly available, we used our dataset to re-fit and generate the weights to use in the analysis. This allowed us to compare across all the signatures as they were all re-fitted similarly. We did not correct for multiple comparisons.

Statistical Analysis:

All analyses were performed with Stata/SE version 17.0 (StataCorp Pty Ltd, College Station, Texas) and R (R version 4.0.2)[37]. We used the pROC package[38] to calculate the AUCs to report the performance of signatures and the DeLong method[39] to compare the AUC values between signatures.

Results

From January 2018 to October 2021, 907 children evaluated for sepsis were enrolled with 595 constituting the discovery, and 312 the RAPIDS validation cohort (**Table 1, eTable 1**). Study samples were obtained at a median of 2.3 (interquartile range (IQR) 1.4, 4.1) hours, and 3.0 (IQR 1.8, 7.5) hours after hospital admission in the discovery, and validation cohort, respectively. Overall, 87 (14.6%) patients in the discovery, and 65 (20.8%) in the validation cohort had organ dysfunction 24-hours after sampling (**eFigure 2**). Of these, 76 (87.4%) patients in the discovery cohort and 57 (87.7%) patients in the validation cohort had organ dysfunction at baseline sampling. 24 (27.6%) patients in the discovery, and 22 (33.8%) patients in the validation cohort developed new or additional organ dysfunction within 24 hours of sampling compared to sampling baseline. 172 (28.9%) and 110 (18.5%) patients in the discovery cohort had DB and DV infections, compared with 63 (20.2%) and 100 (32.1%) in the validation cohort (**eTable 1 and eTable2**).

We assessed differential gene expression in the discovery cohort, first for disease-class, then for disease-severity. Differential expression analysis based on the infection type identified 886 differentially expressed genes (adjusted p-value <0.05) between patients with DV and DB infections (**eFigure 4A; eTable 3**). Comparing patients with versus without organ dysfunction at 24 hours after sampling, 1028 genes were differentially expressed (**eFigure 4B; eTable 3**).

Differentially expressed genes differed based upon whether patients with organ dysfunction had DB or DV infections (**eFigure 4C and 4D; eTable 3**).

Using FSPLS, we discovered a novel 10-gene disease-class signature to distinguish type of infection, which is comprised of *USP18*, *NCF1B*, *BATF*, *CLC*, *S100A11*, *ZBED1*, *PTGES3*, *HLX*, *NOD2* and *ICAM1* genes (**Figure 2A**). This disease-class signature achieved an AUC of 93.5% (95%-CI: 90.5% - 96.6%) in distinguishing DB versus DV in the discovery cohort, an AUC of 94.1% (95%-CI: 90.6% - 97.7%) in the RAPIDS validation cohort (**Figure 2C**), and an AUC of 90.9% (95%-CI: 85.0% - 96.9%) in the EUCLIDS validation cohort (**Table 2**). Similar performances were achieved for other disease-class phenotype comparisons. Compared with previously reported disease-class signatures (**eMethods 7**), this novel signature demonstrated better performance for most classifications (**Table 2, eTable 4**). The disease-class signature also distinguished patients with CBV infection and unknown infection status (**eTable 5**). Gene Ontology enrichment analysis of the novel disease-class signature genes showed enrichment of immune response GO terms (**eMethods 8**, **eFigure 8**).

Using FSPLS, we discovered a novel 10-gene disease-severity signature to identify presence of organ dysfunction 24 hours after sampling which is comprised of *AATBC*, *MAFG*, *VAV1*, *MS4A7*, *IGHA1*, *ATP6V0A1*, *RN7SL3*, *MPP7*, *DSC2* and *PHACTR2* (**Figure 2B**). This signature achieved an AUC of 92.4% (95%-CI: 89.2% - 95.6%) in distinguishing patients with and without organ dysfunction at 24 hours in the discovery cohort and an AUC of 82.2% (95%-CI: 76.3% - 88.1%) in the RAPIDS validation cohort (**Figure 2D; Table 3**). Compared with previously reported gene expression signatures for disease severity (**eMethods 7**) the novel signature demonstrated comparable or superior performance (**Table 3, eTable 6**). Gene Ontology enrichment analysis of the novel disease-severity signature genes showed enrichment

of immunoglobulin complex, signal recognition and proton transporting GO terms indicating biological pathways involved in the development of organ dysfunctions (**eMethods 8**, **eFigure 8**).

To enable prediction of sepsis, we used disease-class stratified severity weights which achieved an AUC of 90.5% (95%-CI 83.3% - 97.6%) and 94.7% (95%-CI 87.9% - 100.0%) in identifying organ dysfunction in patients with predicted DB infection, and predicted DV infection, respectively, in the RAPIDS validation cohort (**Figure 2E and 2F; Table 3**). We then assessed the disease-class and disease-severity signatures using the EUCLIDS cohort, however severity information was only available at the time of sampling. The disease-class stratified severity signatures achieved an AUC of 70.1% (95%-CI 44.1% - 96.2%), and 69.6% (95%-CI 53.1% - 86.0%) in identifying organ dysfunction in children with predicted DB and DV infection, respectively in the EUCLIDS cohort (**Table 3**).

The novel severity signatures performed comparably with identifying secondary severity outcomes at baseline and within 24 hours of sampling, including organ dysfunction remote from the site of infection, type of organ dysfunction, need for organ support, and need for inotrope support (**Table 4**). In the RAPIDS validation cohort, the signatures identified progressive multi-organ dysfunction within 24 hours of sampling with an AUC of 75.8% (95% CI 67.3% - 84.3%) (**Table 4**).

Adding clinical information such as C-reactive protein levels and leukocyte counts to the gene signatures failed to improve the prediction of disease-class and disease-severity, which was superior to routine clinical markers (**eFigure 9**). Both the disease-class and disease-severity signatures in the discovery and validation cohorts performed similarly across the age ranges included (**eTable 7** and **eTable 8**).

Discussion

In this multi-center prospective study involving 912 children evaluated for suspected sepsis, we derived and validated novel gene expression signatures to identify children with confirmed viral versus bacterial infection and organ dysfunction. The sepsis signatures provide actionable information on the likelihood of bacterial (versus viral) infection, and on the likelihood of life-threatening organ dysfunction in 24 hours. Our approach demonstrates the potential of host transcriptomics to distinguish infection types and predict organ dysfunction, as a means to characterize sepsis in children.

In the past years, several infectious disease studies in adult and paediatric patients have investigated host gene expression analyses to differentiate patients with bacterial versus viral infection[9,32,40,41]. At the same time, ICU-based studies revealed pathways and differentially regulated genes associated with mortality in critically ill patients, which may identify patients more likely to suffer harm from specific interventions such as corticosteroids[42,43]. Until recently, however, the integration of the two key dimensions which constitute sepsis (i.e. presence of infection and development of organ dysfunction[1]) by a unifying measure of dysregulated host response has been lacking. In contrast to most previous gene expression studies, which were based on microarray, we utilized RNASeq which provides substantially higher transcript resolution, and we included higher number of patients to increase power. We utilised the FS-PLS approach to find a minimal gene signature for disease class and severity. FS-PLS iteratively finds the next-most explanatory feature after removing the projection of features onto the space spanned by variables previously selected. FS-PLS tends to find smaller signatures than other commonly used approaches, such as LASSO. Furthermore, we enrolled children early upon presentation, with sampling performed at a median of <3 hours after presenting to hospital. Nonetheless, 87.5% of patients who met criteria for organ dysfunction

at 24 hours post admission already manifested at least single organ dysfunction at the time of sampling.

Compared with eight previously reported signatures[9,10,27-32] to diagnose the type of infection, the performance of the novel disease-class signature was similar or higher in terms of AUC in both validation cohorts. Our disease-class signature included 10 transcripts, a number which which has become feasible to implement in rapid point-of-care platforms. Compared with seven previously reported signatures[33-36] to diagnose disease severity, the performance of our novel signature was higher in terms of AUCs in the RAPIDS validation cohort for organ dysfunction 24 hours after sampling associated with bacterial and viral infection. The novel signature was less complex (i.e., 10 genes versus 40 genes) than the best performing previously published severity signature[36]. When assessing other severity outcomes 24 hours after sampling, such as organ dysfunction remote from the site of infection, multi-organ dysfunction, or need for inotropes, the novel disease-severity signature performed well with AUCs above 80%. In the EUCLIDS validation cohort, however, information was only available on organ dysfunction at the time of sampling, and overall AUCs were above 70%. The performance of the severity signature in the EUCLIDS validation may further relate to later sampling, cohort differences such as recruitment bias towards severe bacterial infections, restriction to community-acquired infections and differences in primary clinical focus (**eTable 1**).

Overall, we observed a wider range in diagnostic performance of severity signatures across the discovery and validation cohorts compared with disease-class signatures. Notably, contrary to categorization on microbiologically confirmed bacterial versus viral infection, concepts of severity such as organ dysfunction inherently lack a true gold standard against which to

benchmark biomarkers. Accordingly, the clinical criteria used to define the primary outcome of severity may fall short of the underlying biological complexity, as shown by recent electronic health-record derived studies on sepsis phenotypes[44-47]. The heterogeneity of underlying causes, mechanisms, treatments, and trajectories characterizing critical illness syndromes thus fundamentally challenge the feasibility of a simple severity marker.

Both disease-class and disease-severity gene signatures discovered in this study were shown to predict multiple phenotypes successfully. The disease-class signature identified the infection type in patients evaluated for sepsis. The disease-severity signature identified the presence of organ dysfunction and several other severity phenotypes including whether the organ dysfunction was likely to worsen within 24 hours of sampling. In combination, given the high negative predictive value (eTable 6), the information provided by this novel sepsis signatures has the potential to guide clinical decision-making (rule-out) on use of antimicrobials and escalation of care.

Mortality and other severity outcomes in paediatric sepsis relate directly to delays between presentation and delivery of a sepsis treatment bundle[48]. Sepsis quality improvement programs usually focus on presumed infection in the presence of clinical indicators of altered physiology. However, it is well recognized that clinical features of sepsis are often subtle and non-specific, in particular in children where viral etiologies predominate. Therefore, initiatives to promote early treatment with intravenous antibiotics have been met with criticism as they risk inappropriate use of antibiotics, potentially promoting antimicrobial resistance. In this context, a direct marker of a dysregulated host response to bacterial versus viral infection remains highly desirable and can serve to identify treatable traits early upon presentation.

Several limitations of this study need to be considered. First, although the findings were validated in an a priori defined separate sequencing batch of patients, external validation using the EUCLIDS cohort was only partially feasible, given that 24-hour outcome data had not been collected in the latter. Second, patients were recruited in a high-income setting with a low mortality rate, with a predominance of Caucasian patients, and almost complete absence of fungal and parasitic infections, which may not be representative of patients in less resourced settings. Third, the study design excluded immunosuppressed patients and most included patients had community-acquired infections, implying the need to validate the signature in more comorbid cohorts with hospital-acquired infections. Finally, we did not perform RT-PCR validation of the novel signatures, and future replication using a point-of-care device will be required.

In conclusion, in this large cohort of children evaluated for sepsis encompassing a broad range of disease severity, pathogens, and comorbidities, novel host transcriptomic signatures were able to discriminate patients with bacterial versus viral infection and those who were likely to manifest organ dysfunction within the next 24 hours at high accuracy. Whether such actionable information can direct therapy to patients who are most likely to benefit from timely delivery of a sepsis bundle while reducing unnecessary use of antibiotics, needs to be tested by interventional trials.

Acknowledgements: The authors would like to thank all the parents and children participating in this study and the medical, nursing and research teams at the participating sites for their help in study setup, recruitment, data collection, and monitoring of study data for their invaluable support. We would like to thank Angelika Christ from UQ Sequencing Facility, The University of Queensland for performing all the RNA sequencing work.

Author Contributions: Prof Schlapbach, Dr Ganesamoorthy and Prof Coin have accessed and verified the data, and were responsible for the decision to submit the manuscript. Prof Schlapbach, Dr Ganesamoorthy, Dr Wilson and Prof Coin contributed equally.

Concept and design: Schlapbach, Coin.

Acquisition, analysis, or interpretation of data: Schlapbach, Ganesamoorthy, Wilson, Raman, George, Snelling, Phillips, Irwin, Sharp, La Marsney, Chavan, Hempenstall, Bialasiewicz, MacDonald, Grimwood, Kling, McPherson, Blumenthal, Kaforou, Levin, Herberg, Gibbons, Coin.

Drafting of the manuscript: Schlapbach, Ganesamoorthy, Wilson, Gibbons, Coin.

Critical revision of the manuscript for important intellectual content: Schlapbach, Ganesamoorthy, Wilson, Raman, George, Snelling, Phillips, Irwin, Sharp, Le Marsney, Chavan, Hempenstall, Bialasiewicz, MacDonald, Grimwood, Kling, McPherson, Blumenthal, Kaforou, Levin, Herberg, Gibbons, Coin.

Statistical analysis: Schlapbach, Ganesamoorthy, Le Marsney, Gibbons, Coin.

Obtained funding: Schlapbach, Snelling, Wilson, George, Kaforou, Levin, Herberg, Coin.

Administrative, technical, or material support: Schlapbach, MacDonald, Kling, McPherson, Gibbons.

Supervision: Schlapbach, Herberg, Levin, Gibbons and Coin.

Funding: Research reported in this publication was supported by the Medical Research Future Fund under grant numbers GHFM76734 and MRFF9100000 (LJS). The study has been further funded by grants from the Children`s Hospital Foundation, Brisbane, Australia (LJS, AB, SR); Children`s Hospital Foundation, Brisbane, Australia (DG); Gold Coast Hospital Foundation (SG); Emergency Medicine Foundation, Brisbane, Australia (PS); Far North Queensland

Foundation, Cairns, Australia (LJS); Townsville Hospital and Health Services SERTA Grant (AC); Brisbane Diamantina Health Partners, Brisbane, Australia (LJS); Australian Infectious Diseases Research Centre, Brisbane, Australia (AB, LJS). LJS received a Practitioner Fellowship from the National Health and Medical Research Council Australia, and support from the NOMIS foundation. CW was supported by a Wellcome Trust Grant (203928/Z/16/Z). AB acknowledges support by an Australian Research Council Future Fellowship (FT220100487). MK is supported by the Wellcome Trust (206508/Z/17/Z) and the Medical Research Foundation (MRF-160-0008-ELP-KAFO-C0801). CW, MK, ML and JH receive support from the NIHR Imperial BRC. The EUCLIDS consortium was supported by European Union's 7th Framework program (EC-GA 279185). None of the funding bodies had any involvement in study design, conduct, data collection, analysis, interpretation, writing of the manuscript, and the decision to submit.

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Conflict of interest: None of the authors declared a conflict of interest.

Data availability: RNA sequencing data, processed gene counts and patient metadata are available at EGA (link will be provided after the paper is accepted for publication).

References

1. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016; **315**(8): 801-10.

2. Rudd KE, Johnson SC, Agesa KM, et al. Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study. *Lancet* 2020; **395**(10219): 200-11.

3. Carlton EF, Barbaro RP, Iwashyna TJ, Prescott HC. Cost of Pediatric Severe Sepsis Hospitalizations. *JAMA Pediatr* 2019; **173**(10): 986-7.

4. Maddux AB, Zimmerman JJ, Banks RK, et al. Health Resource Use in Survivors of Pediatric Septic Shock in the United States. *Pediatr Crit Care Med* 2022; **23**(6): e277-e88.

5. Schlapbach LJ, Weiss SL, Wolf J. Reducing Collateral Damage From Mandates for Time to Antibiotics in Pediatric Sepsis-Primum Non Nocere. *JAMA Pediatr* 2019; **173**(5): 409-10.

6. Angus DC. Opening the Debate on the New Sepsis Definition Defining Sepsis: A Case of Bounded Rationality and Fuzzy Thinking? *Am J Respir Crit Care Med* 2016; **194**(1): 14-5.

7. Cohen J, Vincent JL, Adhikari NK, et al. Sepsis: a roadmap for future research. *Lancet Infect Dis* 2015; **15**(5): 581-614.

8. Reyes M, Filbin MR, Bhattacharyya RP, et al. An immune-cell signature of bacterial sepsis. *Nat Med* 2020; **26**(3): 333-40.

9. Herberg JA, Kaforou M, Wright VJ, et al. Diagnostic Test Accuracy of a 2-Transcript Host RNA Signature for Discriminating Bacterial vs Viral Infection in Febrile Children. *JAMA* 2016; **316**(8): 835-45.

10. McHugh L, Seldon TA, Brandon RA, et al. A Molecular Host Response Assay to Discriminate Between Sepsis and Infection-Negative Systemic Inflammation in Critically Ill Patients: Discovery and Validation in Independent Cohorts. *PLoS Med* 2015; **12**(12): e1001916.

11. Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ* 2015; **351**: h5527.

12. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing

translational research informatics support. *Journal of biomedical informatics* 2009; **42**(2): 377-81.

13. Goldstein B, Giroir B, Randolph A, International Consensus Conference on Pediatric S. International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics. *Pediatr Crit Care Med* 2005; **6**(1): 2-8.

14. Gebara BM. Values for systolic blood pressure. *Pediatr Crit Care Med* 2005; **6**(4): 500; author reply -1.

15. Schlapbach LJ, Goertz S, Hagenbuch N, et al. Organ Dysfunction in Children With Blood Culture-Proven Sepsis: Comparative Performance of Four Scores in a National Cohort Study. *Pediatr Crit Care Med* 2023.

16. Morin L, Hall M, de Souza D, et al. The Current and Future State of Pediatric Sepsis Definitions: An International Survey. *Pediatrics* 2022; **149**(6).

17. Levin M, Cunnington AJ, Wilson C, et al. Effects of saline or albumin fluid bolus in resuscitation: evidence from re-analysis of the FEAST trial. *Lancet Respir Med* 2019; **7**(7): 581-93.

18. Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed 5/2/2023 2023).

19. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 2016; **32**(19): 3047-8.

20. Buderer NM. Statistical methodology: I. Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity. *Acad Emerg Med* 1996; **3**(9): 895-900.

21. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013; **29**(1): 15-21.

22. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* 2015; **31**(2): 166-9.

23. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; **15**(12): 550.

24. Boeddha NP, Schlapbach LJ, Driessen GJ, et al. Mortality and morbidity in community-acquired sepsis in European pediatric intensive care units: a prospective cohort study from the European Childhood Life-threatening Infectious Disease Study (EUCLIDS). *Crit Care* 2018; **22**(1): 143.

25. Martinon-Torres F, Salas A, Rivero-Calle I, et al. Life-threatening infections in children in Europe (the EUCLIDS Project): a prospective cohort study. *Lancet Child Adolesc Health* 2018; **2**(6): 404-14.

26. Nijman RG, Oostenbrink R, Moll HA, et al. A Novel Framework for Phenotyping Children With Suspected or Confirmed Infection for Future Biomarker Studies. *Front Pediatr* 2021; **9**: 688272.

27. Tang BM, Shojaei M, Parnell GP, et al. A novel immune biomarker IFI27 discriminates between influenza and bacteria in patients with suspected respiratory infection. *Eur Respir J* 2017; **49**(6).

28. Wong HR, Salisbury S, Xiao Q, et al. The pediatric sepsis biomarker risk model. *Critical Care* 2012; **16**(5).

29. Sweeney TE, Wong HR, Khatri P. Robust classification of bacterial and viral infections via integrated host gene expression diagnostics. *Sci Transl Med* 2016; **8**(346): 346ra91.

30. Sampson DL, Fox BA, Yager TD, et al. A Four-Biomarker Blood Signature Discriminates Systemic Inflammation Due to Viral Infection Versus Other Etiologies. *Sci Rep* 2017; **7**(1): 2914.

31. Li Y, Li Y, Bai Z, Pan J, Wang J, Fang F. Identification of potential transcriptomic markers in developing pediatric sepsis: a weighted gene co-expression network analysis and a case-control validation study. *Journal of translational medicine* 2017; **15**(1): 254.

32. Li HK, Kaforou M, Rodriguez-Manzano J, et al. Discovery and validation of a three-gene signature to distinguish COVID-19 and other viral infections in emergency infectious disease presentations: a case-control and observational cohort study. *Lancet Microbe* 2021; **2**(11): e594-e603.

33. Lukaszewski RA, Jones HE, Gersuk VH, et al. Presymptomatic diagnosis of postoperative infection and sepsis using gene expression signatures. *Intensive Care Med* 2022; **48**(9): 1133-43.

34. Pena OM, Hancock DG, Lyle NH, et al. An Endotoxin Tolerance Signature Predicts Sepsis and Organ Dysfunction at Initial Clinical Presentation. *EBioMedicine* 2014; **1**(1): 64-71.

35. Irwin AD, Grant A, Williams R, et al. Predicting Risk of Serious Bacterial Infections in Febrile Children in the Emergency Department. *Pediatrics* 2017; **140**(2): e20162853.

36. Baghela A, Pena OM, Lee AH, et al. Predicting sepsis severity at first clinical presentation: The role of endotypes and mechanistic signatures. *EBioMedicine* 2022; **75**: 103776.

37. Team RC. R: A Language and Environment for Statistical Computing 2022. https://www.R-project.org/.

38. Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 2011; **12**: 77.

39. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988; **44**(3): 837-45.

40. Tsalik EL, Henao R, Montgomery JL, et al. Discriminating Bacterial and Viral Infection Using a Rapid Host Gene Expression Test. *Crit Care Med* 2021; **49**(10): 1651-63.

41. Sweeney TE, Azad TD, Donato M, et al. Unsupervised Analysis of Transcriptomics in Bacterial Sepsis Across Multiple Datasets Reveals Three Robust Clusters. *Crit Care Med* 2018; **46**(6): 915-25.

42. Sweeney TE, Perumal TM, Henao R, et al. A community approach to mortality prediction in sepsis via gene expression analysis. *Nat Commun* 2018; **9**(1): 694.

43. Wong HR, Cvijanovich NZ, Anas N, et al. Developing a clinically feasible personalized medicine approach to pediatric septic shock. *Am J Respir Crit Care Med* 2015; **191**(3): 309-15.

44. Maslove DM, Tang B, Shankar-Hari M, et al. Redefining critical illness. *Nature Medicine* 2022; **28**(6): 1141-8.

45. Komorowski M, Green A, Tatham KC, Seymour C, Antcliffe D. Sepsis biomarkers and diagnostic tools with a focus on machine learning. *eBioMedicine* 2022; **86**.

46. Seymour CW, Kennedy JN, Wang S, et al. Derivation, Validation, and Potential Treatment Implications of Novel Clinical Phenotypes for Sepsis. *JAMA* 2019; **321**(20): 2003-17.

47. Sanchez-Pinto LN, Bennet TD, Stroup EK, et al. Derivation, Validation, and Clinical Relevance of a Pediatric Sepsis Phenotype With Persistent Hypoxemia, Encephalopathy, and Shock. *Pediatr Crit Care Med* 2023; **24**(10): 795-806.

48. Weiss SL, Peters MJ, Alhazzani W, et al. Surviving sepsis campaign international guidelines for the management of septic shock and sepsis-associated organ dysfunction in children. *Intensive Care Med* 2020; **46**(Suppl 1): 10-67.

Figure Legends:

Figure 1. Schematic workflow of the multi-phenotype signature discovery using transcriptomics data. The discovery cohort was used for the novel signature discovery. The disease-class signature and disease-severity signature were discovered using the FSPLS method. These signatures were validated on two independent validation cohorts. First, the infection type of the sample was predicted using the disease-class signature, then the probability of developing organ dysfunction was predicted using the disease-severity signature. DB – Definite Bacterial; DV – Definite Viral; PV- Probable Viral; PB – Probable Bacterial; NI – Non-infectious; OD – Organ Dysfunction; ROC curve – Receiver Operating Characteristics curve; AUC – Area Under the Curve.

Figure 2: Performance of disease-class signature and disease-severity signature in distinguishing infection type and identifying organ dysfunction. Heat map showing the expression of **(A)** disease-class signature genes across patients in the discovery cohort with definite bacterial (n=172) and definite viral (n=110) infections; **(B)** disease-severity signature genes across patients in the discovery cohort with organ dysfunction (n=87) versus without organ dysfunction (n=508) at 24-hours after sampling; Receiver Operating Characteristics (ROC) curve for the performance of the signature in the discovery (red lines) and validation (blue lines) data to distinguish **(C)** definite bacterial versus definite viral infections; **(D)** with versus without organ dysfunction in all the patients; **(E)** with versus without organ dysfunction in patients with predicted definite bacterial infections; and **(F)** with versus without organ dysfunction in patients with predicted definite viral infections. Continuous red and blue lines indicate AUC and the dashed lines shows the respective 95% confidence interval.

Table 1. Clinical, microbiological, and severity characteristics of the discovery and validation cohorts of children evaluated for sepsis.

Characteristic	Category	Discovery N=595	RAPIDS Validation N=312	EUCLIDS Validation N=362
Gender *n (%)*	Female	278 (46.7)	124 (39.7)	189 (52.2)
Age *n (%)*	<1 year	156 (26.2)	43 (13.8)	99 (27.4)
	1-5 years	217 (36.5)	159 (51.0)	152 (42.0)
	5-10 years	119 (20.0)	56 (18.0)	60 (16.6)
	10-18 years	103 (17.3)	54 (17.3)	51 (14.1)
Age (years) *median (IQR)*		2.8 (1.0, 7.7)	3.4 (1.4, 7.3)	2.6 (0.8, 5.7)
Chronic condition *n (%)*	Any	132 (22.2)	92 (29.5)	-
	Asthma	27 (4.5)	12 (3.9)	-
	Congenital Malformation	21 (3.5)	16 (5.1)	-
	Congenital Heart Defect	20 (3.4)	20 (6.4)	-
	Cerebral Palsy, Severe Encephalopathy	18 (3.0)	14 (4.5)	-
	Syndrome or Genetic Disorder	0 (0)	22 (7.1)	-
	Other Chronic Condition	80 (13.5)	68 (21.8)	-
Symptoms at presentation *n (%)*	Fever	464 (78.0)	242 (79.6)	-
	Rash	71 (11.9)	49 (16.1)	-
	Altered level of consciousness	56 (9.4)	38 (12.5)	-
	Irritability	92 (15.5)	35 (11.5)	-
	Seizures	34 (5.7)	25 (8.2)	-
	Pain	155 (26.1)	96 (31.6)	-
	Nausea/Vomiting	182 (30.6)	97 (31.9)	-
	Diarrhoea	72 (12.1)	28 (9.2)	-
	Respiratory distress/apnoea	141 (23.7)	63 (20.7)	-
	Cough	207 (34.8)	97 (31.9)	-
	Pale/cyanotic episode	49 (8.2)	27 (8.9)	-

Characteristic	Category	Discovery N=595	RAPIDS Validation N=312	EUCLIDS Validation N=362
	Cold extremities	13 (2.2)	8 (2.6)	-
	Skin / wound infection	36 (6.1)	12 (4.0)	-
	Other	156 (26.2)	71 (23.4)	-
Primary clinical focus *n (%)*	Sepsis without a source	165 (27.7)	94 (30.1)	58 (16.0)
	Lower respiratory infection	183 (30.8)	86 (27.6)	105 (29.0)
	Upper respiratory infection	46 (7.7)	32 (10.3)	4 (1.1)
	Meningitis /Encephalitis	18 (3.0)	9 (2.9)	66 (18.2)
	Urinary tract infection	47 (7.9)	20 (6.4)	13 (3.6)
	Arthritis/ Osteomyelitis	15 (2.5)	3 (1.4)	17 (4.7)
	Skin infection	28 (4.7)	7 (2.2)	25 (6.9)
	Wound infection	15 (2.5)	5 (1.6)	0
	Toxic shock syndrome	6 (1.0)	2 (0.6)	11 (3.0)
	ENT infection/abscess	29 (4.9)	11 (3.5)	4 (1.1)
	Gastroenteritis	18 (3.0)	18 (5.8)	5 (1.4)
	Other	25 (4.2)	25 (8.0)	54 (14.9)
Time from hospital admission to sampling (hours) *median (IQR)*		2.3 (1.4, 4.1)	3.0 (1.8, 7.5)	-
Admission to PICU *n (%)*	Yes	173 (29.1)	92 (29.5)	212 (58.6)
Patients with length of stay of under 24 hours *n* (%)		137 (23.0)	82 (26.3)	
Laboratory characteristics at baseline *median (IQR)*	Base excess [mmol/l]	-2.1 (-4.7, -0.2) *(N=379)*	-1.8 (-4.3, 0.2) *(N=178)*	-4.4 (-7.1, -1.3) *(N=192)*
	paO_2 [mmHg]	99 (70, 130) *(N=89)*	81 (69, 106) *(N=55)*	97 (73, 139) *(N=84)*
	pCO_2 [mmHg]	38 (34, 45) *(N=89)*	40 (36, 46) *(N=109)*	45 (35, 56) *(N=183)*
	Lactate [mmol/l]	1.5 (1.1, 2.3) *(N=394)*	1.4 (1.0, 2.2) *(N=207)*	1.4 (0.9, 2.3) *(N=185)*

Characteristic	Category	Discovery N=595	RAPIDS Validation N=312	EUCLIDS Validation N=362
	Creatinine [µmol/l]	30 (30, 44) *(N=574)*	31 (30, 42) *(N=283)*	36 (27, 49) *(N=303)*
	Bilirubin [µmol/l]	7 (5, 12) *(N=569)*	7 (5, 12) *(N=287)*	6 (4, 100) *(N=206)*
	International Normalized Ratio	1.3 (1.1, 1.6) *(N=140)*	1.3 (1.2, 1.7) *(N=69)*	1.4 (1.2, 1.9) *(N=90)*
	Fibrinogen [g/L]	3.4 (2.6, 5.2) *(N=137)*	3.3 (2.4, 4.1) *(N=69)*	-
	Platelets [$*10^3$/µL]	303 (219, 378) *(N=564)*	270 (198, 363) *(N=291)*	255 (163, 347) *(N=338)*
	White Cell Count [$*10^3$/µL]	11.7 (7.9, 16.5) *(N=583)*	10.7 (7.1, 15.9) *(N=295)*	-
	C-reactive protein [mg/L]	25 (7, 95) *(N=531)*	34 (10, 89) *(N=267)*	-
Infection Type *n (%)*	Definite Bacterial	172 (28.9)	63 (20.2)	190 (52.5)
	Probable Bacterial	64 (10.8)	39 (12.5)	60 (16.6)
	Definite Viral	110 (18.5)	100 (32.1)	39 (10.8)
	Probable Viral	87 (14.6)	32 (10.3)	12 (3.3)
	Combined Bacterial /Viral Infection	64 (10.8)	30 (9.6)	1 (0.3)
	Non-Infectious Illness	45 (7.6)	36 (11.9)	-
	Unknown	53 (8.9)	9 (2.9)	60 (16.6)
Deceased *n (%)*	Death	6 (1.0)	4 (1.3)	9 (2.5)
At least one organ dysfunction *n (%)*	Baseline	134 (22.5)	76 (24.4)	200 (55.3)
	24 hours	87 (14.6)	65 (20.8)	
Organ dysfunction remote from the primary site of infection *n (%)*	Baseline	132 (22.11)	74 (23.7)	179 (49.5)
	24 hours	86 (14.4)	61 (19.6)	

Characteristic	Category	Discovery N=595	RAPIDS Validation N=312	EUCLIDS Validation N=362
Any organ support *n (%)*	Baseline	74 (12.4)	51 (16.4)	164 (45.3)
	24 hours	69 (11.6)	41 (13.1)	
Any Inotropes *n (%)*	Baseline	41 (6.9)	28 (9.0)	111 (30.7)
	24 hours	46 (7.7)	26 (8.3)	
Multi-organ dysfunction *n (%)*	Baseline	81 (13.6)	50 (16.0)	136 (37.6)
	24 hours	68 (11.4)	40 (12.8)	

Table 2: Performance of the novel disease-class gene expression signature in distinguishing infection types, compared to previously published host transcriptomic signatures. Areas under the Curve (AUC) with corresponding 95%-confidence intervals (CI) are shown. For each tested phenotype, the best performing signature in terms of AUC is highlighted in red.

Phenotype	Signature	Number of genes in signature	Discovery (n=595)				RAPIDS Validation (n=312)				EUCLIDS Validation (n=362)			
			AUC	95% CI low	95% CI high	P-value *	AUC	95% CI low	95% CI high	P-value *	AUC	95% CI low	95% CI high	P-value*
Definite Bacterial versus Definite Viral	Novel disease-class Signature	10	**0.935**	0.905	0.966	-	**0.941**	0.906	0.977	-	0.909	0.850	0.969	-
	Herberg et al[9]	2	0.861	0.815	0.908	0.183	0.900	0.856	0.945	0.475	**0.923**	0.887	0.959	0.848
	McHugh et al[10]	4	0.788	0.733	0.843	0.019	0.750	0.673	0.827	0.024	0.738	0.646	0.831	0.120
	Tang et al[27]	1	0.894	0.857	0.931	0.391	0.883	0.830	0.936	0.362	0.895	0.843	0.948	0.858
	Wong et al[28]	5	0.828	0.779	0.876	0.060	0.773	0.699	0.848	0.041	0.687	0.591	0.784	0.503
	Sweeney et al[29]	7	0.924	0.894	0.953	0.779	0.911	0.865	0.956	0.594	0.911	0.862	0.960	0.980
	Sampson et al[30]	4	0.894	0.853	0.935	0.414	0.894	0.845	0.944	0.438	0.921	0.875	0.966	0.880
	Li et al[31]	4	0.691	0.627	0.754	0.001	0.668	0.576	0.761	0.006	0.800	0.731	0.870	0.235
	Li et al (2021)[32]	3	0.881	0.837	0.925	0.307	0.907	0.860	0.954	0.555	0.906	0.855	0.956	0.964

Definite Bacterial versus Probable Viral	Novel disease-class Signature	10	**0.912**	0.876	0.948	-	0.863	0.771	0.954	-	**0.935**	0.899	0.972	-
	Herberg et al[9]	2	0.794	0.738	0.849	0.072	**0.872**	0.785	0.959	0.940	0.887	0.811	0.963	0.565
	McHugh et al[10]	4	0.788	0.730	0.846	0.069	0.712	0.596	0.829	0.311	0.696	0.526	0.866	0.168
	Tang et al[27]	1	0.722	0.656	0.788	0.011	0.788	0.682	0.893	0.592	0.726	0.588	0.864	0.142
	Wong et al[28]	5	0.770	0.711	0.830	0.040	0.829	0.739	0.919	0.793	0.838	0.750	0.926	0.309
	Sweeney et al[29]	7	0.862	0.817	0.908	0.390	0.850	0.764	0.935	0.918	0.770	0.642	0.899	0.216
	Sampson et al[30]	4	0.760	0.696	0.824	0.038	0.852	0.761	0.943	0.935	0.831	0.697	0.964	0.450
	Li et al[31]	4	0.708	0.643	0.773	0.006	0.779	0.677	0.881	0.540	0.754	0.597	0.911	0.262
	Li et al (2021)[32]	3	0.810	0.755	0.866	0.122	0.851	0.757	0.944	0.927	0.844	0.737	0.951	0.421
Definite Viral versus Probable Bacterial	Novel disease-class Signature	10	**0.909**	0.864	0.953	-	**0.856**	0.784	0.929	-	0.793	0.695	0.891	-
	Herberg et al[9]	2	0.805	0.736	0.873	0.201	0.827	0.756	0.898	0.772	0.762	0.668	0.855	0.816
	McHugh et al[10]	4	0.741	0.666	0.817	0.057	0.765	0.666	0.865	0.460	0.668	0.554	0.782	0.404
	Tang et al[27]	1	0.833	0.768	0.897	0.333	0.797	0.707	0.887	0.607	0.877	0.810	0.944	0.480
	Wong et al[28]	5	0.780	0.709	0.850	0.122	0.686	0.592	0.781	0.154	0.544	0.422	0.665	0.109
	Sweeney et al[29]	7	0.869	0.810	0.927	0.588	0.839	0.763	0.915	0.871	**0.889**	0.824	0.954	0.414
	Sampson et al[30]	4	0.838	0.777	0.900	0.352	0.836	0.762	0.910	0.845	0.807	0.720	0.894	0.917
	Li et al[31]	4	0.603	0.517	0.690	0.002	0.564	0.452	0.677	0.029	0.578	0.465	0.691	0.150
	Li et al (2021)[32]	3	0.828	0.764	0.892	0.303	0.841	0.767	0.916	0.885	0.774	0.680	0.868	0.887
Definite Bacterial versus Non-Infectious	Novel disease-class Signature	10	**0.917**	0.879	0.954	-	0.654	0.540	0.768	-	NA			
	Herberg et al[9]	2	0.775	0.704	0.847	0.081	0.571	0.454	0.689	0.614				
	McHugh et al[10]	4	0.830	0.766	0.893	0.239	0.663	0.554	0.771	0.957				
	Tang et al[27]	1	0.598	0.504	0.691	0.002	0.462	0.346	0.578	0.239				

	Wong et al[28]	5	0.702	0.617	0.787	0.021	0.712	0.607	0.817	0.709	
	Sweeney et al[29]	7	0.841	0.773	0.909	0.332	0.678	0.574	0.783	0.874	
	Sampson et al[30]	4	0.701	0.612	0.790	0.026	0.551	0.434	0.668	0.529	
	Li et al[31]	4	0.786	0.716	0.857	0.104	**0.713**	0.609	0.818	0.701	
	Li et al (2021)[32]	3	0.615	0.526	0.704	0.002	0.479	0.362	0.596	0.283	
Definite Viral versus Non-Infectious	Novel disease-class Signature	10	**0.945**	0.904	0.985	-	**0.796**	0.709	0.882	-	NA
	Herberg et al[9]	2	0.909	0.862	0.957	0.570	0.771	0.677	0.865	0.849	
	McHugh et al[10]	4	0.850	0.788	0.913	0.203	0.690	0.578	0.801	0.454	
	Tang et al[27]	1	0.914	0.869	0.960	0.615	0.749	0.647	0.850	0.726	
	Wong et al[28]	5	0.821	0.745	0.897	0.151	0.694	0.594	0.793	0.440	
	Sweeney et al[29]	7	0.933	0.896	0.970	0.834	0.766	0.669	0.862	0.819	
	Sampson et al[30]	4	0.856	0.785	0.927	0.277	0.752	0.656	0.849	0.738	
	Li et al[31]	4	0.862	0.797	0.927	0.280	0.726	0.623	0.829	0.604	
	Li et al (2021)[32]	3	0.857	0.795	0.919	0.233	0.754	0.659	0.848	0.744	

* P-value for comparison between the AUCs for the Novel Signature and other reported signatures.

Table 3: Performance of novel severity signature in identifying organ dysfunction for different infection types, compared to previously published host transcriptomic signatures. Areas under the Curve (AUC) with corresponding 95%-confidence intervals (CI) are shown. For each tested phenotype, the best performing signature in terms of AUC is highlighted in red.

Phenotype	**Signature**	**Number of genes in signature**	**Discovery (n=595)**				**RAPIDS Validation (n=312)**				**EUCLIDS Validation (n=362)**			
			AUC	**95% CI low**	**95% CI high**	**P-value***	**AUC**	**95% CI low**	**95% CI high**	**P-value***	**AUC**	**95% CI low**	**95% CI high**	**P-value***

OD at 24hrs	Novel disease-severity Signature	10	**0.924**	0.892	0.956	-	0.822	0.763	0.881	-	NA
	Irwin et al[35]	3	0.755	0.695	0.815	0.013	0.772	0.708	0.837	0.571	
	Lukaszewski et al[33]	27	0.870	0.831	0.910	0.295	0.802	0.742	0.862	0.810	
	Pena et al[34]	31	0.865	0.814	0.917	0.333	0.750	0.675	0.825	0.449	
	Baghela et al[36]	40	0.892	0.851	0.933	0.543	0.787	0.724	0.849	0.681	
	Baghela et al - Severity[36]	8	0.851	0.798	0.904	0.242	0.724	0.650	0.798	0.301	
	Baghela et al - CR[36]	12	0.849	0.800	0.898	0.204	**0.846**	0.790	0.902	0.767	
	Baghela et al - Mortality[36]	10	0.844	0.798	0.890	0.154	0.822	0.765	0.879	0.997	
OD at 24hrs in Predicted Bacterial Infection	Novel disease-severity Signature	10	0.940	0.876	1.000	-	**0.905**	0.833	0.976	-	NA
	Irwin et al[35]	3	0.792	0.711	0.874	0.150	0.735	0.593	0.877	0.287	
	Lukaszewski et al[33]	27	0.887	0.820	0.953	0.561	0.761	0.624	0.899	0.354	
	Pena et al[34]	31	0.942	0.897	0.988	0.972	0.709	0.559	0.860	0.241	
	Baghela et al[36]	40	**0.989**	0.977	1.000	0.439	0.788	0.649	0.928	0.457	
	Baghela et al - Severity[36]	8	0.914	0.844	0.985	0.788	0.713	0.574	0.852	0.218	
	Baghela et al - CR[36]	12	0.909	0.838	0.980	0.747	0.859	0.761	0.956	0.704	
	Baghela et al - Mortality[36]	10	0.858	0.775	0.941	0.432	0.611	0.409	0.813	0.170	
OD at 24hrs in PredictedViral Infection	Novel disease-severity Signature	10	**0.944**	0.885	1.000	-	**0.947**	0.879	1.000	-	NA
	Irwin et al[35]	3	0.847	0.751	0.943	0.385	0.895	0.776	1.000	0.683	

	Lukaszewski et al[33]	27	0.883	0.795	0.971	0.559	0.865	0.706	1.000	0.604				
	Pena et al[34]	31	0.864	0.741	0.988	0.559	0.740	0.541	0.938	0.318				
	Baghela et al[36]	40	0.917	0.813	1.000	0.803	0.877	0.789	0.965	0.512				
	Baghela et al - Severity[36]	8	0.843	0.704	0.982	0.500	0.758	0.563	0.952	0.353				
	Baghela et al - CR[36]	12	0.920	0.857	0.983	0.773	0.837	0.657	1.000	0.546				
	Baghela et al - Mortality[36]	10	0.852	0.760	0.945	0.400	0.824	0.643	1.000	0.514				
OD at 0hrs	Novel disease-severity Signature	10	**0.852**	0.809	0.895	-	**0.775**	0.712	0.838	-	0.775	0.727	0.823	-
	Irwin et al[35]	3	0.725	0.672	0.777	0.060	0.724	0.656	0.791	0.580	0.786	0.739	0.832	0.874
	Lukaszewski et al[33]	27	0.806	0.762	0.850	0.457	0.771	0.706	0.836	0.964	0.791	0.745	0.837	0.813
	Pena et al[34]	31	0.808	0.763	0.852	0.473	0.770	0.708	0.831	0.953	0.794	0.748	0.840	0.771
	Baghela et al[36]	40	0.825	0.782	0.868	0.657	0.751	0.686	0.816	0.793	**0.821**	0.777	0.864	0.481
	Baghela et al - Severity[36]	8	0.785	0.735	0.834	0.304	0.725	0.653	0.797	0.600	0.811	0.766	0.855	0.584
	Baghela et al - CR[36]	12	0.772	0.720	0.823	0.229	0.715	0.645	0.786	0.531	0.792	0.746	0.838	0.796
	Baghela et al - Mortality[36]	10	0.744	0.692	0.796	0.109	0.763	0.699	0.828	0.898	0.787	0.740	0.833	0.860
OD at 0hrs in Predicted Bacterial Infection	Novel disease-severity Signature	10	0.924	0.867	0.981	-	**0.886**	0.802	0.970	-	0.701	0.441	0.962	-
	Irwin et al[35]	3	0.804	0.721	0.888	0.236	0.748	0.594	0.901	0.428	0.771	0.572	0.970	0.832
	Lukaszewski et al[33]	27	0.898	0.837	0.959	0.754	0.824	0.701	0.946	0.674	0.667	0.420	0.913	0.923
	Pena et al[34]	31	0.903	0.841	0.964	0.799	0.728	0.572	0.884	0.372	0.708	0.453	0.964	0.985
	Baghela et al[36]	40	**0.972**	0.944	1.000	0.456	0.728	0.568	0.888	0.382	0.743	0.522	0.964	0.903

	Baghela et al - Severity[36]	8	0.852	0.766	0.938	0.484	0.782	0.657	0.907	0.490	0.611	0.341	0.881	0.810
	Baghela et al - CR[36]	12	0.863	0.784	0.943	0.535	0.740	0.603	0.878	0.365	0.660	0.379	0.940	0.913
	Baghela et al - Mortality[36]	10	0.857	0.789	0.926	0.454	0.707	0.533	0.881	0.355	**0.882**	0.722	1.000	0.541
OD at 0hrs in Predicted Viral Infection	Novel disease-severity Signature	10	0.828	0.753	0.902	-	0.757	0.588	0.925	-	0.696	0.531	0.860	-
	Irwin et al[35]	3	0.729	0.637	0.821	0.403	**0.844**	0.676	1.000	0.709	0.679	0.509	0.849	0.944
	Lukaszewski et al[33]	27	0.803	0.733	0.874	0.812	0.779	0.645	0.914	0.916	0.776	0.630	0.923	0.714
	Pena et al[34]	31	0.809	0.721	0.897	0.872	0.804	0.703	0.905	0.810	0.781	0.644	0.917	0.691
	Baghela et al[36]	40	**0.869**	0.802	0.935	0.683	0.596	0.409	0.782	0.522	**0.925**	0.854	0.997	0.201
	Baghela et al - Severity[36]	8	0.784	0.704	0.864	0.690	0.723	0.550	0.896	0.888	0.861	0.752	0.970	0.402
	Baghela et al - CR[36]	12	0.732	0.640	0.824	0.417	0.790	0.639	0.941	0.884	0.776	0.636	0.917	0.709
	Baghela et al - Mortality[36]	10	0.741	0.653	0.828	0.448	0.622	0.404	0.840	0.626	0.820	0.697	0.943	0.546

* P-value for comparison between the AUCs for the Novel Signature and other reported signatures.

Table 4: Performance of novel severity signature in identifying various severity outcomes. Areas under the Curve (AUC) with corresponding 95%-confidence intervals (CI) are shown for each outcome assessed 24 hours after study blood sampling ("24hrs"), and at time of study blood sampling ("0hrs"). Organ dysfunction was assessed using the 2005 International Pediatric Sepsis Consensus Conference criteria.

Phenotype	Discovery (n=595)			RAPIDS Validation (n=312)			EUCLIDS Validation (n=362)		
	AUC	95% CI low	95% CI high	AUC	95% CI low	95% CI high	AUC	95% CI low	95% CI high
OD remote to infection site 24hrs	0.927	0.894	0.959	0.820	0.759	0.882		NA	
OD remote to infection site 0hrs	0.854	0.811	0.897	0.778	0.714	0.842	0.770	0.730	0.820
Need for organ support 24hrs	0.957	0.940	0.974	0.825	0.751	0.899		NA	
Need for organ support 0hrs	0.925	0.895	0.954	0.781	0.709	0.853	0.780	0.740	0.830
Administration of Inotropes 24hrs	0.963	0.946	0.981	0.846	0.759	0.932		NA	
Administration of Inotropes 0hrs	0.940	0.890	0.980	0.820	0.730	0.910	0.830	0.780	0.870
CVS, Resp, or CNS OD 24hrs^	0.962	0.943	0.980	0.828	0.732	0.924		NA	
CVS, Resp, or CNS OD 0hrs^	0.919	0.874	0.963	0.852	0.757	0.947	0.730	0.660	0.814
MOD 24hrs	0.959	0.942	0.976	0.858	0.792	0.925		NA	
MOD 0hrs	0.902	0.861	0.943	0.801	0.729	0.873	0.800	0.750	0.850
OD Better at 24hrs[#]	0.715	0.648	0.783	0.623	0.515	0.731		NA	
OD Worse at 24hrs[#]	0.872	0.810	0.934	0.721	0.611	0.831		NA	
MOD Better at 24hrs*	0.798	0.708	0.888	0.645	0.501	0.788		NA	
MOD Worse at 24hrs*	0.896	0.851	0.941	0.758	0.673	0.843		NA	
Cardiac OD 24hrs	0.958	0.937	0.978	0.811	0.736	0.886		NA	
Cardiac OD 0hrs	0.837	0.783	0.892	0.789	0.705	0.872	0.810	0.770	0.860
Respiratory OD 24hrs	0.953	0.934	0.972	0.840	0.771	0.909		NA	
Respiratory OD 0 hrs	0.914	0.882	0.947	0.782	0.705	0.860	0.760	0.700	0.810
Neurologic OD 24hrs	0.908	0.861	0.955	0.820	0.750	0.890		NA	
Neurologic OD 0 hrs	0.885	0.840	0.930	0.806	0.737	0.875	0.680	0.610	0.750

Renal OD 24hrs	0.890	0.777	1.000	0.692	0.366	1.000		NA	
Renal OD 0hrs	0.864	0.773	0.955	0.692	0.462	0.921	0.760	0.660	0.860
Hematologic OD 24hrs	0.945	0.906	0.985	0.735	0.594	0.876		NA	
Hematologic OD 0hrs	0.869	0.800	0.938	0.793	0.650	0.935	0.700	0.580	0.820
Hepatic OD 24hrs	0.950	0.887	1.000	0.678	0.102	1.000		NA	
Hepatic OD 0hrs	0.749	0.410	1.000	0.600	0.279	0.921	0.720	0.410	1.000

^ Presence of either cardiac, respiratory or neurological organ dysfunction
# Compared to OD at time of sampling, OD increasing (worse) or decreasing (better) at 24-hours post sampling
* Compared to MOD at time of sampling, MOD increasing to >=2 (worse) or decreasing to 0/1 (better) at 24-hours post sampling
OD - organ dysfunction; CVS - cardiovascular; Resp - respiratory; CNS - central nervous system; MOD - multi organ dysfunction; AUC – Area Under the Curve; CI – Confidence Interval
NA – data not available