U.S. Department of Health and Human Services

National Institute of General Medical Sciences

Second NIGMS Sepsis R21/R33 Biospecimens Investigators' Meeting

March 11, 2025

Virtual Meeting

Abstracts

Contents

Session I: Patient Recruitment, Sample Collection, and Data Integration

The Sepsis ClinicAl Resource And Biorepository (SCARAB) Project

Lorraine B. Ware, M.D., Julie A. Bastarache, M.D., Vanderbilt University Medical Center

SCARAB is a two-center prospective cohort of adult and pediatric sepsis patients and controls with a robust complement of biospecimens, detailed syndromic phenotyping, and a user-friendly web-based study portal to facilitate sharing with external investigators. SCARAB is designed to be both comprehensive and easily scalable to large multicenter implementation. The study prospectively enrolls subjects from pediatric and adult ICUs at Vanderbilt University Medical Center and Meharry Medical College. Subjects are enrolled in the first 24 hours of their ICU stay, and serial biologic specimens of blood, DNA, respiratory micro-droplets [from Heat Moisture Exchange (HME) filters], tracheal aspirates, and urine are collected. To study the end organ most commonly injured in sepsis, we have established best practices for the collection, processing, and storage of lung fluid collected from HME filters, a novel method for sampling the distal airspace in ventilated patients. The study has just entered its final year of R33 funding. Current enrollment from the combined R21/R33 phases includes 414 patients (379 adults, 35 children). Working with our collaborators in Biomedical Informatics, we have developed and are validating automated phenotyping algorithms to identify patients with sepsis and organ failures, including ARDS, acute kidney injury, and delirium. We are using cutting-edge natural language processing methods, such as concept mapping, to conduct detailed sub-phenotyping for clinically important variables that are challenging to phenotype on a large scale, such as delirium phenotyping in the absence of standard assessments. As we approach our target enrollment, we are working to make SCARAB clinical data and biospecimens accessible to the broader research community through our web-based study portal and have convened a Resource Utilization Committee comprising study investigators and national experts in sepsis and organ dysfunction. In order to maximize utilization and encourage requests from young investigators, there is no fee (other than shipping costs) to request or receive samples from SCARAB. In addition, we are finalizing our state-of-the-art data de-identification methods to conform to the most rigorous security standards.

Developing a Scalable, Multicenter Pediatric Sepsis Biorepository and Clinical Database

Fran Balamuth, M.D., Ph.D., Children's Hospital of Philadelphia

Objective: In the R21 phase of the study, we successfully demonstrated the feasibility of collecting and processing pre-resuscitation blood samples for immune phenotyping, bioenergetic profiling, and plasma proteomics in children presenting with sepsis to the emergency department (ED). In the initial part of the project, we demonstrated that we could achieve high-end yields of PBMCs and plasma volumes from 5 ml of blood, with adequate yields in as little as 2 ml. Additionally, we demonstrated that samples processed after 12 hours of storage had similar PBMC yield and quantitative analyses results as samples processed within 3 hours. These findings make collection and processing very feasible for multicenter studies, particularly in sites without access to PBMC processing around the clock, which is one of the major contributions of the R21 phase. Based on these results, we tested an updated protocol and enrolled 39 patients at CHOP, successfully obtaining pre-resuscitation samples from 92% of patients, above our threshold for success. We had a limited collection of subsequent samples due to parental hesitancy and early discharges. However, post-resuscitation samples were a secondary aim of the project.

In parallel to the specimen collection, we developed and validated a data harmonization and quality assurance pipeline for clinical and biorepository metadata extracted from the electronic health record (EHR) and centralized in a cloud server at Lurie. We collected detailed information on all 2,046 patient encounters that triggered a sepsis alert in the EDs at CHOP and Lurie during the R21 phase, including the subset who had specimens collected. This population provides information regarding the clinical characteristics and

timing of sepsis cases in the emergency department, which can be leveraged to optimize specimen collection strategies. Additionally, the detailed clinical information collected on patients with specimens will allow the development of a cohort discovery application during the R33 phase.

Summary of Achievements in the R33 Phase for Year 3: In Year 3 of the R33 phase, we have worked successfully with the biorepository at CHOP to build an easily reproducible infrastructure for receiving samples with coded identifiers from external sites that can be linked to the clinical database of patients. We have now established a system that includes barcoded storage tubes and a detailed accessioning template that sites are able to complete, which contains sample metadata as well as the same hashed MRN (i.e., de-identified) that is being used for clinical data transfer to the Lurie secure cloud server. In addition, we have worked with external sites to share the PBMC processing protocols optimized during the R21 phase at CHOP and ensure expertise at all sites in these methods. We have also been able to develop off-hours plasma processing capabilities using 2 distinct mechanisms at CHOP and Colorado, both of which could be transferable to additional future sites. This involves collaboration with the 24h clinical lab services available at CHOP and adding capacity for off-hours ED-based research staff to process samples in Colorado. CHOP restarted enrollment for biospecimen collection for the R33 phase on 1/4/25 using the new barcoded systems that the external sites will use and has enrolled 4 patients as of 1/24/25. Colorado is finalizing procedures and plans to start enrolling in February 2025, and Lurie will follow in March 2025. The delays encountered were largely due to the team needing to spend time addressing issues of identification/coding of samples, and we feel confident that this was time well spent, as we now have a system that could be reproducible and scaled across multiple sites in the future.

The clinical data transfer system and analytic pipeline in the Lurie cloud server are now well established for CHOP and Lurie data, and we anticipate transferring new data from CHOP beginning in March 2025 once the data has clinically matured for the R33 phase patients (at least 60 days from enrollment start). The initial data transfer from Colorado is anticipated in May 2025.

Single Cell Transcriptome Assessment of Blood and Lower Airways From Critically Ill COVID-19 Patients

Leopoldo N. Segal, M.D., M.S., New York University School of Medicine

OBJECTIVES:
The COVID-19 pandemic had a profound impact on human health, particularly among critically ill patients who experience the highest mortality. Transcriptomic data can provide a comprehensive assessment of the immune regulation occurring in different compartments. One of the key aspects of the NYU Biorepository is to utilize scRNAseq approaches to endotype critically-ill SARS-CoV-2-infected patients seeking signatures in the systemic and lower airway, site of disease, and compartment. While scRNAseq is increasingly utilized, few studies have used this approach on lower airway cells from acute COVID-19 patients. In addition, the need for cryopreservation and the high abundance of neutrophils impose additional technical challenges in these samples. Here, we aimed to test the performance of different methods of sample processing and sequencing.

METHODS:
Three methods were predominantly tested using cryopreserved PBMCs and lower airway cells isolated from critically ill COVID-19 patients, which were thawed and washed using culture medium. For cell hashing, samples were tagged with Cell Multiplexing Oligo (CMO), which is a barcoded oligonucleotide conjugated to lipid and can be used to label individual cells; then, labeled cells can be pooled for library prep. In addition, we tried Chromium Fixed RNA Profiling. Briefly, cryopreserved BAL cells were thawed, filtered, and then

fixed with 4% formaldehyde for 1 hour. Fixed and permeabilized cells were hybridized with probes, and library prep was performed using the Chromium Fixed RNA Profiling for Multiplexed samples method. Library prep was performed by using Chromium Next GEM Single Cell 3' V3.1 (Dual index) protocol, which includes 3 major steps: Gem Generation and barcoding, Post GEM-RT cleanup and cDNA amplification, 3' gene expression library construction. 15,000 cells of each sample were taken for single 10X cell sequencing.

RESULTS:
We initially attempted to use a cost-effective approach based on scRNAseq with cell hashing. For this approach, since cell viability is critical for the performance, we first tested this using PBMCs. In the cell-hashed library preparation,> 50% cells were doublets. This supports that after staining with CMO, washes of each sample were not successful at completely removing free CMO, thereby contaminating the other samples upon pooling. This data suggests that a CMO approach would perform even worse with lower airway samples. We then tried Chromium Fixed RNA Profiling on lower airway cells obtained via bronchoscopy, since this assay is robust to samples at much lower viability. We selected 4 BALCs from critically ill COVID patients. The Chromium Fixed RNA Profiling libraries tested prior to sequencing showed low yield. Despite this, we still attempted to perform 10X sequencing. After sequence analysis, we found that all four samples had severe quality issues with extremely low cell numbers; 3 out of 4 had recovered cell numbers less than 100 single-cell transcriptomes. Finally, we performed scRNAseq without hashing or fixation, which demonstrated a much better yield. Using this method, we are able to obtain quality data in more than 1,000 cells per sample in 5/9 of BAL samples (> 50%), which is the most challenging sample type. In contrast, all PBMC samples achieved this threshold. Further, analyses of individual samples (focusing on the more challenging BAL samples) showed that we consistently achieve < 20% of mitochondrial RNA per single cell transcriptome. The BAL cell data shown here demonstrates that, for the most part, we are achieving data on >1,000 distinct genes with sufficient depth in the order of what's commonly used as the target for scRNAseq using other biological samples (most of which are less challenging to work with than these cryopreserved lower airway samples).

CONCLUSION:
Our analyses outline the challenges of performing single-cell transcriptomic assays on cryopreserved lower airway samples and provide a roadmap for how to successfully generate quality data on these samples.

The Scientific Value of Premature Infant Biospecimens Collection

Stephanie Prescott, Ph.D., A.P.R.N., N.N.P.-B.C., University of South Florida and Inova Children's Hospital

OBJECTIVES:
Our aim is to collect biospecimens before, during, and after the onset of neonatal sepsis, ultimately to establish predictive and discriminatory models. We intend to determine the most useful biospecimens, the best timing for collection, and the most reliable collection and storage practices. Our first objective was to successfully enroll neonates within the first days of life. Our second goal was to determine the most reliable sample collection and storage practices.

METHODS:
Recruitment and Collection - Establish a committee of nurses to assist with patient identification, sample collection, and communication with the research team. Approach families for enrollment before maternal discharge from post-partum. Develop a cheat sheet for sample collection. Create a parent letter with information about the study. Display a poster with enrollment goals and numbers visible to parents and staff. Design a "superbaby" poster to hang on the doors of participating babies with acknowledgement of their contribution. Create an orderset to remind nurses of impending sample collections. Create a timeline

for each patient on a header in the chart. Daily patient rounding to discuss the status of the sample collections.

Sample Processing and Storage - Rectal swab vs. stool (frozen immediately vs. room temp). Stool preserved in ETOH vs lysis buffer for DNA extraction. Saliva cotton swab vs. Flox swab vs. passive drool for protein analysis (cortisol). RNA extraction from frozen whole blood.

RESULTS:
Recruitment and Collection-Initial slow start because of Single IRB, contractual agreements, MTDA agreements, individual site IRBs, translation services, and inexperience of the research team. There was also competition between studies for the same patient population, and a historically low census at one of the sites. It was very difficult to obtain consent if parents were not approached while still admitted to the hospital. There were also funding freezes due to a PI position change and recent government actions, which stalled contractual agreements. In the first year, we only achieved 15 enrollments, but in the first half of the second year, we have been able to enroll 73 patients and are averaging 3-5 enrollments per week.

Sample Processing and Storage- Stool collection and extraction methods yielded similar results to the gold standard, with enrichment of skin species in rectal swab collections. Flox swabs reliably reproduced results from passive drool. Flox swabs yielded lower quality and quantity of DNA, but sufficient for sequencing purposes. RNA extraction from frozen whole blood protocols is still pending.

CONCLUSION:
Optimizing enrollment success requires approaching parents after the initial shock of NICU admission, but before maternal discharge from the hospital. Optimizing sample collection success depends on a group of individuals dedicated to frequent contact with the nursing staff using multiple methods of communication—visual, verbal, and written. Stool may be collected and frozen immediately or stored in ETOH or lysis buffer up to 7 days at room temperature. Flox swabs yield a lower quantity and quality of DNA but remain within acceptable range for sequencing. Flox swabs are valid alternatives to passive drool collection for analyzing salivary cortisol.

Leveraging Multi-Omics to Maximize the Scientific Value of Pediatric Sepsis Biorepository and Advance Patient Endotyping

Mihir R. Atreya[1,2], Chengkun Yang[3], Scarlett Ripberger[1], Kelli Harmon[1], Stephen W. Standage[1,2], Andrew J. Lautz[1,2], Nelson-Sanchez Pinto[4], Rishikesan Kamaleswaran[5]
Affiliations:
1. Division of Critical Care Medicine, Cincinnati Children's Hospital Medical Center.
2. Department of Pediatrics, University of Cincinnati.
3. Department of Biomedical Engineering, Duke University.
4. Division of Critical Care Medicine, Biomedical Informatics, Lurie Children's Hospital, Northwestern University.
5. Departments of Surgery, Duke University.

OBJECTIVES:
Pediatric sepsis is a major cause of morbidity and mortality, yet its molecular heterogeneity remains poorly understood. We sought to leverage a multi-omics approach to enhance an existing pediatric sepsis biorepository and improve patient endotyping. By integrating paired and temporally sampled whole blood transcriptomic and DNA methylation data, we seek to determine molecular pathways linked to sepsis subclasses and clinically relevant outcomes.

METHODS:
We utilized existing whole blood-derived biospecimens from pediatric patients meeting consensus criteria for septic shock recruited through a multi-center IRB-approved prospective observational cohort. Matched and temporally paired DNA and RNA samples, collected on days 1 and 3, were processed under rigorous quality control standards. For RNAseq, we analyzed temporally paired RNAseq data from 202 patients. Additionally, non-sequenced RNA samples underwent stringent quality control, requiring a 260/280 ratio of 1.9–2.1, RIN > 6, and a concentration > 20 ng/μL. DNA samples were included if they met a 260/280 ratio of 1.8–2.0 to ensure purity. Raw DNA methylation 850k array data were generated from 106 patients and underwent data quality control to ensure integrity and reliability.

RESULTS:
We identified 215 pediatric septic shock patients with high-quality temporally paired RNA specimens in addition to previously sequenced samples. 432 patients were identified to have high-quality DNA specimens of whom we generated raw DNA methylation array data. The IDAT files generated underwent stringent batch normalization, removal of cross-reactive probes, and those associated with sex chromosomes. Subsequent steps will include identification of differentially methylated regions (DMRs) associated with clinically relevant outcomes, including a composite of death or presence of >2 organ dysfunctions and biomarker-based risk strata. In addition, the MultiPower algorithm will be used to determine an appropriate sample size for future integration of datasets and to facilitate downstream multi-omic analyses.

CONCLUSION:
This study established a robust framework for integrating transcriptomic and epigenomic data in pediatric septic shock, facilitating the identification of molecular features linked to disease heterogeneity and clinical outcomes. The generation of high-quality RNAseq and DNA methylation data from temporally paired samples provides a valuable resource for future analyses. Ongoing work will focus on defining DMRs and gene expression signatures that distinguish biologically relevant patient subsets. These findings will contribute to the refinement of multi-omic endotypes, enhancing predictive enrichment strategies and informing precision medicine approaches in pediatric sepsis.

Scalable and Interoperable Framework for a Clinically Diverse and Generalizable Sepsis Biorepository Using Electronic Alerts for Recruitment Driven by Artificial Intelligence (SIBER-AI)

Annette Esper, M.D., M.Sc., Rishi Kamaleswaran, Ph.D., Carmen Polito, M.D., Neal Dickert, M.D., Ph.D., Peter Hesketh, Ph.D., Emory University School of Medicine and Georgia Institute of Technology

The SIBER-AI aims to create a comprehensive sepsis biorepository at earlier time points during sepsis onset. The objectives of the study were the following: 1) to adapt a clinical sepsis screening algorithm to support research collection of a multi-modal clinical, physiologic, volatilomic, and multi-omic biorepository profile among enriched sepsis populations; 2) to design and test novel biospecimen collection among enriched sepsis populations in both ambulance and hospital environments, including the emergency department, intensive care unit and hospital wards; and 3) to develop novel approaches to consent that reflect clinical context of sepsis, maximize representativeness, and enhance trust and engagement among patients and surrogate decision-makers. We developed a screening algorithm that successfully identifies patients with sepsis, and achieved a base AUROC of 0.88, with a sensitivity of 0.76 and PPV of 0.59 in our retrospective analysis. In a prospective deployment of the algorithm, we identified 45 cases for enrollment in a prospective validation by a board-certified critical care intensivist. Eighteen of the 45 cases (40%) were correctly identified with sepsis within 24 hours of the initial firing of the alert. We have successfully

implemented a case-series to develop and test the most feasible and reliable volatile compounds from the skin of patients and ambient air, which we believe aids more indirect measurements of patient condition and senses the acute deterioration of medical condition beforehand. These efforts and achievements show the promising collection, determination, and utilization to identify sepsis patients early. We met with EMS leadership to develop a prehospital protocol to test environmental VOCs (volatile organic compounds) located in ambient air samples. Through multiple planning meetings, we learned that EMS encounters in Atlanta are brief due to an urban environment and relatively short transport times. Air collection into Tedlar bags, as originally planned, requires a higher collection time than EMS on-scene times. Consequently, this approach was not feasible and led us to investigate alternative strategies. Finally, we completed formative interviews with patients and surrogates regarding consent approaches to the sepsis biorepository and were able to identify key themes regarding the consent process in the critically ill. *Key themes* include: general acceptance of deferred consent approaches when needed; preference for early communication and notification; and emphasis on reducing invasiveness and communicating privacy protections. We have also constituted and partnered with a patient advisory community to optimize our approach. *Key changes* include: development of a simple, graphics-based information sheet that can serve to notify patients about the study and will include a QR code linking to a video that will explain the biorepository study and add a more human face to the study; re-worked consent form and key information sheet removing complex and extraneous information, focusing on what participation involves, clarifying the value of the study, and avoiding language that unnecessarily fosters mistrust or invite misperceptions of risk.

Session II: Novel Samples, Biomarkers, and Analytic Techniques

Multiomic, Mass Spectrometry-based Analysis of Dried Blood for Deep Phenotyping of Sepsis

Matt Foster, Ph.D., Duke University School of Medicine

OBJECTIVES:
Volumetric microsampling (VAMS) of whole blood has the potential for easy and convenient biospecimen collection and storage. In contrast to plasma/serum, whole blood may suffer less pre-analytical variability and better capture relevant changes in circulating factors for phenotyping of disease. Mass spectrometry is a powerful tool for the quantification of protein abundances, post-translational modifications (e.g., glycosylation and phosphorylation), and metabolites. We are developing methods for mass spectrometry-based analysis of whole blood captured by VAMS and are applying these multi-omic approaches to phenotyping of sepsis biobanks.

METHODS:
Venous-EDTA whole blood was captured on 20 microliter Mitra devices from the Sepsis Characterization in Tanzania (SICK; days 0, 1, 3, 28 after hospital admission with Sepsis-2 criteria) and red blood cell ATP and Transfusion in Sepsis (STAR; Sepsis-3 and healthy controls, single time-point) studies. Mitra tips were processed in 96-well formats, using serial trypsin digestion, N-glycopeptide and phosphopeptide enrichment, followed by liquid chromatography coupled to tandem mass spectrometry. Analysis of unenriched proteomes and phosphoproteomes used data-independent acquisition, and analysis of N-glycoproteomes used stepped-collision energy data-dependent acquisition. Data were analyzed using Spectronaut, Glyco-Decipher, and R.

RESULTS:

Approximately 2,000 proteins, 9,000 glycopeptides, and 8,000 phosphopeptides were quantified in dried blood from 130 SICK and STAR study samples, and additional quality controls. Linear regression analysis showed high correlation between clinical laboratory values from SICK subjects at day 0 (e.g., C-reactive protein and urea) and blood proteins, including acute phase reactants (CRP, SAA1/2) and markers of kidney disease (CYTC, COLA1). Further, the universal vital assessment (UVA) score positively correlated with phospho-OPN and negatively correlated with glyco-CLUS. Resolution of inflammation between day 0-28 was suggested by decreases in neutrophil (phospho)proteins. Multi-omic factor analysis of STAR subjects demonstrated clustering of sepsis patients from healthy controls based on proteins, N-glycopeptides, and phosphopeptides.

CONCLUSION:
Collection of whole blood on Mitra devices can be readily incorporated into ongoing biobanking in studies of sepsis. Multi-omic analysis can be accomplished with streamlined sample prep and high-throughput LC-MS/MS workflows. Disease and resolution are reflected in numerous cellular and secreted factors and correlate with clinical/laboratory data, potentially identifying endotypes and therapeutic targets. Additional work in progress is addressing orthogonal analyses (phosphoTyr enrichment, metabolomics) and scaling to a post-COVID biobank to investigate molecular determinants of functional recovery.

Cryo-PRO Facilitates Whole Blood Cryopreservation for scRNAseq of Immune Cells from Clinical Samples

Roby Bhattacharyya, M.D., Ph.D., Michael Filbin, M.D., Harvard Medical School and Broad Institute

OBJECTIVES:
Single-cell RNA sequencing (scRNAseq) of peripheral blood mononuclear cells (PBMCs) has enhanced our understanding of host immune mechanisms in small cohorts, particularly in diseases with complex and heterogeneous immune responses such as sepsis. However, PBMC isolation from blood requires technical expertise, training, and approximately two hours of onsite processing using Ficoll density gradient separation (Ficoll) for scRNAseq compatibility, precluding large-scale sample collection at most clinical sites.

METHODS:
To minimize onsite processing, we developed Cryo-PRO (Cryopreservation with PBMC Recovery Offsite), a method of PBMC isolation from cryopreserved whole blood that allows immediate onsite sample cryopreservation and subsequent PBMC isolation in batch in a central laboratory via magnetic bead-based depletion and flow cytometry enrichment prior to scRNAseq.

RESULTS:
We compared scRNAseq results from samples processed using Cryo-PRO versus standard onsite Ficoll separation in 32 samples from 23 patients with sepsis. Quality control metrics for scRNAseq data were highly similar between methods, as were critical scRNAseq outputs including cell substate fractions and marker genes across multiple cell types and substates, including an important monocyte substate enriched in patients with sepsis (Pearson correlation 0.78, $p<0.001$; 70% of top marker genes shared). Unlike hybridization-based methods from fixed cells, Cryo-PRO captured the diversity of T-cell receptor (TCR) sequences, producing comparable results from targeted TCR sequencing to the standard Ficoll method.

CONCLUSION:

Cryo-PRO reduced onsite sample processing time from >2 hours to <15 minutes and was reproducible across two enrollment sites, thus demonstrating potential for expanding scRNAseq in multicenter studies of sepsis and other diseases.

Redox Trapping for Biospecimen Preservation and Innovation in Sepsis Care

Cristina M. Furdui, Ph.D., D. Clark Files, M.D., Wake Forest University School of Medicine

OBJECTIVES:
Blood is the most used biospecimen for diagnostics and the discovery of new biomarkers for early disease detection and treatment. It is rapidly accessible, easily handled, and versatile for various applications. However, its utility in assessing redox biomarkers of disease is often compromised due to artifactual oxidation during processing and storage, leading to the loss of specimen molecular integrity. To enhance our understanding of disease pathophysiology, reduce pre-analytical variability, improve patient staging, and aid in the development of new therapies, we have developed and tested a new formulation (RMX) as an alternative to the most used EDTA or heparin blood collection methods.

METHODS:
Blood from healthy donors was used to evaluate RMX capacity for preserving the endogenous redox state of proteins and maintaining sample stability, particularly by assessing the redox state of peroxiredoxins and glutathione and the degradation of vascular endothelial growth factor (VEGF) over time and through repeated freeze/thaw cycles. Additionally, blood from sepsis patients was utilized to determine RMX compatibility with standard clinical assays [e.g., comprehensive metabolic panel (CMP), complete blood count (CBC)].

RESULTS:
The data demonstrate that RMX provides a rapid and effective quenching of redox state without interfering with the commonly used clinical laboratory protocols. Notably, quenched samples remained stable during storage and through repeated freeze-thaw cycles up to a month. This innovative approach to mitigating artifactual oxidation now allows for accurate redox state measurements and enhances the quantitative analytical precision of biomarker measurements in clinical specimens. This was demonstrated by improvements in the analysis of lactate, a critical diagnostic metabolite in sepsis patients.

CONCLUSION:
The RMX formulation holds significant potential for identifying patient heterogeneity and tracking disease progression, thereby improving clinical staging and precision, and supporting the development of new therapeutic targets across diseases.

Dimethylmethylene Blue as a Rapid Assay for Circulating Glycosaminoglycans in Sepsis

Eric P. Schmidt, M.D., Max Kravitz, John Lee, Alicia Rizzo, Nathan Shapiro, M.D., Massachusetts General Hospital and Harvard Medical School

OBJECTIVES:
The endothelial glycocalyx is the innermost layer of all blood vessels. It is composed of glycosaminoglycans and proteoglycans. The most abundant glycosaminoglycan in the endothelial glycocalyx is heparan sulfate (HS). In sepsis, endothelial glycocalyx degradation, as evidenced by mass spectrometry (MS) quantification of HS in blood, is strongly associated with increased mortality. There are currently no available assays for the rapid quantification of circulating HS in the emergency department. The objective of this investigation is

to study dimethylmethylene blue (DMMB) as a rapid assay for the detection of HS in plasma and urine by assessing its correlation with plasma HS measured by MS in blood and urine samples of sepsis patients.

METHODS:
This is a prospective, observational cohort study of a convenience sample of patients in a tertiary care emergency department. Inclusion criteria: age ≥ 18 years, clinical evidence of infection, and Sequential Organ Failure Assessment (SOFA) score ≥ 4. Exclusion criteria: an alternative diagnosis to infection as the etiology of illness, and inability to obtain consent. DMMB assay: A Colorimetric assay was performed on urine and plasma samples. Urine creatinine assay was run on urine samples to adjust for variance in urine concentration. MS: "Gold standard comparator" was assayed for HS on plasma samples. We compared blood HS levels measured with MS to levels obtained by urine and plasma DMMB. Pearson correlation coefficient, with an alpha of 0.05 and 95% Confidence Interval (CI), was used to assess the correlation between DMMB and MS measurements.

RESULTS:
Two hundred nineteen patients were enrolled in this study. Two hundred sixteen patients had baseline plasma samples available. Eighty-three patients had 24-hour plasma samples available. One hundred fifty patients had baseline urine samples available. The following correlations were observed: plasma DMMB-plasma MS: r=0.47 (95% CI: 0.37 to 0.55, p<0.0001), urine DMMB/urine creatinine-plasma MS: r=0.75 (95% CI: 0.67 to 0.81, p<0.0001)

CONCLUSION:
Urine DMMB, when controlled for urine creatinine, correlated strongly with mass spectrometry measures of plasma HS. Plasma DMMB and plasma HS had a moderate correlation. Future studies are ongoing to determine clinical factors associated with urine and/or plasma DMMB accuracy (or inaccuracy) as a point-of-care assay for circulating HS.

Biobank of Small Extracellular Vesicles for Pediatric Sepsis: A Liquid Biopsy for Unraveling Heterogeneity and Molecular Mechanisms

Basilia Zingarelli, M.D., Ph.D., Jennifer Kaplan, M.D., M.S., Division of Critical Care Medicine, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine

INTRODUCTION:
Small extracellular vesicles (EVs) are nanoparticles (30-150 nm) secreted by various cell types. They play a crucial role in intercellular communication by transporting bioactive molecules such as proteins, lipids, and nucleic acids (including RNA and DNA) between cells. This cargo can influence various physiological and pathological processes, making EVs valuable for diagnostic and therapeutic applications. The goal of our project was to establish a biobank of EVs derived from serum and plasma samples of children with sepsis. This biobank aims to provide a valuable resource for studying disease mechanisms, identifying biomarkers, and developing therapeutic strategies.

OBJECTIVES:
Specific objectives were 1) to establish the most effective method of EV isolation from small amounts of frozen plasma and serum samples of existing biorepositories in the setting of a research laboratory; 2) to provide evidence that EVs are suitable for functional studies in readout *in vitro* assays; 3) to provide evidence that EVs are suitable for high-throughput analysis of RNA cargo.

METHODS:

De-identified frozen plasma and serum samples of 48 children (>30 days to 18 years old) were obtained from the Cincinnati Children's Hospital Medical Center Discover Together Biobank (control non-septic patients) and the Critical Care Medicine Biorepository (patients with sepsis). All samples were collected under Institutional Review Board-approved protocols. We compared two technologies for EV isolation, ultracentrifugation and a phosphatidylserine (PS)-affinity method. Efficiency, size distribution, and concentration of EVs were determined by nanoparticle tracking analysis; criteria of efficiency of RNA recovery rate, purity, and RNA integrity were also described. A standardized and quantifiable *in vitro* assay was developed using stable-transfected human THP1 reporter monocytes as a readout assay to evaluate EV inflammatory functions.

RESULTS:
The PS-affinity method demonstrated superior efficiency, yielding a higher concentration of EVs with a more consistent size distribution compared to ultracentrifugation in both serum and plasma samples. Independent of the extraction method, higher concentrations of EVs were counted in serum samples compared to plasma samples, which aligns with the observation that retrieved vesicles are consistently more abundant in serum than in plasma. The concentration of serum-derived EVs was significantly higher in patients with sepsis when compared with control children, independently of the extraction method. The concentration of plasma-derived EVs was significantly higher in patients with sepsis when compared with control children, only when the PS-affinity method was used. Both extraction methods were effective for screening in *in vitro* cell systems and for RNA extraction, facilitating downstream molecular analyses.

CONCLUSION:
Our results show that the pre-analytical factors have a significant impact on the quantification and composition of EVs isolated from plasma and serum. The PS-affinity method enables a high yield of EV concentration and effectively identifies significant differences in sepsis. On the other hand, a major limitation of the ultracentrifugation method is its lower capacity to allow extraction when using very small volumes of serum or plasma. Nevertheless, both methods allow isolation of EVs with similar physical and functional characteristics. Our data also provide evidence that the establishment of a biobank of EVs, coupled with optimized extraction methods and workflow standardization, provides a robust platform for pediatric disease research, enabling comprehensive studies on EV biology and their potential clinical applications. By leveraging these optimized protocols, our future retrospective and prospective clinical studies will determine whether functional characteristics and transcriptomic cargo profiles by RNAseq of plasma or serum-derived EVs will reflect different "endotypes" of sepsis with distinct clinical presentations.

Establishment of a Multi-Center Biobank of Patient-Specific Induced Pluripotent Stem Cells (iPSCs) for Pediatric Sepsis Research

Mihir R. Atreya[1,3], Oguzhan Tezel[1], Shuangmin Zhang[1], Kelli Harmon[1], Amy Pitstick[2], Christopher Mayhew[2,3], Basilia Zingarelli[1,3], Andrew J. Lautz[1,3]
Affiliations:
1. Division of Critical Care Medicine, Cincinnati Children's Hospital Medical Center.
2. Pluripotent Stem Cell Facility, Division of Developmental Biology, Cincinnati Children's Hospital Medical Center.
3. Department of Pediatrics, University of Cincinnati.

OBJECTIVES:
Pediatric sepsis remains a major cause of morbidity and mortality, with limited therapeutic advancements due to biological heterogeneity among patients. Precision medicine has identified sepsis subtypes, but the lack of human-relevant models hinders mechanistic research and translation. iPSCs offer a renewable source

of human cells and a platform to study patient-specific responses. Yet, the feasibility of iPSC collection among critically ill patients is unproven. We sought to optimize cell collection, enrichment, and reprogramming strategies to establish iPSC biobanking for pediatric sepsis to enable downstream mechanistic studies.

METHODS:
We conducted a nested single-center observational cohort study of patients aged 0–18 years meeting consensus criteria for pediatric septic shock. Patients aged 10–18 years were preferentially dual consented for iPSC generation and biobanking. Single-cell fractions were isolated using a streamlined immunomagnetic enrichment protocol (AutoMACS), including post-erythrocyte depletion, immune (CD45+), and circulating endothelial cells (CD45-/CD34+). Enrichment quality was confirmed via flow cytometry. The post-erythrocyte depletion fraction was reprogrammed into iPSCs at the institutional pluripotent stem cell facility. Patient-specific iPSC lines underwent rigorous quality control and differentiation into two distinct lineages using established protocols (Stem Cell Research).

RESULTS:
We recruited 13 patients with a median age of 14.5 years. Cellular enrichment was performed successfully with a median collection of 2 $X10^7$ cells/ml, 8 $X10^6$ CD45+ cells/ml, and 2.7 $X10^5$ CD45-/CD34+ cells/ml. Flow cytometry demonstrated enrichment >90%. Eight distinct patient-specific iPSC lines were successfully generated from a post-RBC depletion fraction that passed quality control. Patient-specific iPSC lines were differentiated into iPSC-monocytes and cardiomyocytes. Functional characterization, whole genome sequencing, and multi-omic assessment of patient-specific iPSC-derived cells stimulated with risk-stratified plasma derived from patients is ongoing.

CONCLUSION: Our findings demonstrate the feasibility of cellular enrichment and iPSC generation from critically ill pediatric sepsis patients. Successful differentiation into monocytes and cardiomyocytes supports their use as potential disease models for systemic and organ-specific pathobiology. Future efforts will expand biobanking and validate findings across centers. Ultimately, patient-specific iPSCs could enable organoid models to study sepsis-induced tissue dysfunction and serve as robust models for targeted therapies.

Session III: Updates from R21 Sepsis Biospecimens Investigators – Abstracts for Non-Presenters

Inertial Microfluidics Enables Functional Analysis of Neutrophils Isolated From Ultra-Low Blood Volume Samples

Roberto Rodriguez-Moncayo, Stephanie Pons, Luciana P. Tavares, Hyungkook Jeon, John-Alexander Preuss, Janina Bahnemann, Jongyoon Han, Bruce D. Levy, Joel Voldman, Brigham and Women's Hospital and Massachusetts Institute of Technology

Monitoring immune cell function is increasingly being recognized as a more relevant biomarker than traditional white blood cell counts, yet the need for repeated relatively large blood volumes still continues to pose a significant challenge. To overcome this, we developed a sample-sparing platform using inertial microfluidics that can process as little as 10µL of blood to isolate leukocytes for downstream functional analysis. Our platform isolates leukocytes with ~80% purity, >90% in-device recovery, and >95% viability. Neutrophils were our primary focus due to their sensitivity to external stimuli and their critical role in immune responses. Neutrophils isolated through our new method did not show inadvertent activation, as

evidenced by unchanged expression of activation markers CD62L and CD11b, with phenotypes comparable to control cells in whole blood. We conducted a range of functional assays, including phagocytosis, ROS production, and NETosis, with all tests confirming that neutrophils maintained their functionality after isolation. These assays were performed using standard laboratory workflows, demonstrating the platform's compatibility with techniques such as flow cytometry and cell culture assays. Furthermore, we showed the versatility of our platform by successfully isolating leukocytes from challenging samples, including mouse blood from the vena cava or tail vein, as well as human capillary blood obtained by fingerstick. This adaptability highlights the potential of this platform for clinical and research applications, particularly in frequent immune monitoring or cases where sample volume is limited.

Comparison of Sepsis Biomarkers Derived from Remnant and Research-Collected Biospecimens

Arnab Chowdhury[1], Kelly Urbanek[1], Octavia Peck Palmer[3], Emma Baumgartner[1], Stacy Wendell[2], Steven Mullett[2], Jason N. Kennedy[1], Derek Angus[1], Christopher W. Seymour[1]

Affiliations:

1. University of Pittsburgh, Department of Critical Care Medicine.
2. University of Pittsburgh School of Medicine, Department of Pharmacology and Chemical Biology.
3. University of Pittsburgh, Department of Pathology.

OBJECTIVES:

Sepsis is a leading cause of mortality and morbidity globally. For mechanistic research in sepsis, biospecimens are obtained from patients with costly, time-intensive methods. In the Remnant Investigation in Sepsis (REMISE) study, we sought to measure the agreement of candidate biomarker values from bedside research coordinator-collected specimens compared to measurements from remnant biospecimens. Little is also known about the performance of multi-omic assays, such as metabolomic or lipidomic measurements in remnant plasma.

METHODS:

We performed a prospective cohort study of 48 adult patients who presented with Sepsis-3 criteria within six hours of emergency department arrival. Remnant biospecimens were obtained from first clinical blood draws, and coordinator-collected biospecimens were collected after standard research procedures. We analyzed 7 biomarkers—heme oxygenase-1 (HO-1), interleukin-6 (IL-6), pentraxin-3, e-selectin, p-selectin, angiopoietin-1, angiopoietin-2-using the Wilcoxon Rank Sum test. Agreement was determined using Spearman's rank coefficient. Untargeted metabolomic and lipidomic analyses were performed with a 2-sided t-test (p=0.01).

RESULTS:

Among 48 sepsis patients, the mean age was 53±16 years, 67% were men, and the mean SOFA score was 3.5±1.9. For protein biomarkers, we found no differences in the paired analysis of Angiopoietin-2 and E-Selectin. We observed statistically significant, but clinically minimal differences between HO-1, IL-6, pentraxin-3, p-selectin, and angiopoietin-1. There was a strong monotonic correlation for 4 of 7 biomarkers [HO-1, IL-6, angiopoietin-2, e-selectin (Spearman's rank coefficient >0.07, p<0.001)]. For untargeted metabolomics, we observed that 13.6% of the 4,750 putatively identified metabolites were statistically different between bedside and remnant samples. The pathways differentially enriched were predominantly those of lineolate and CoA catabolism. Of 7,686 lipid species identified, a smaller proportion were different between remnant and coordinator collected samples (1.7%).

Phage Diversity in Cell-Free DNA Identifies Bacterial Pathogens in Human Sepsis Cases

Paul Bollyky, M.D., Ph.D., Samuel Yang, M.D., Stanford University

Bacteriophages, viruses that infect bacteria, have great specificity for their bacterial hosts at the strain and species level. However, the relationship between the phageome and associated bacterial population dynamics is unclear. Here, we generated a computational pipeline to identify sequences associated with bacteriophages and their bacterial hosts in cell-free DNA from plasma samples. Analysis of two independent cohorts, including a Stanford Cohort of 61 septic patients and 10 controls, and the SeqStudy cohort of 224 septic patients and 167 controls, reveals a circulating phageome in the plasma of all sampled individuals. Moreover, infection is associated with over-representation of pathogen-specific phages, allowing for the identification of bacterial pathogens. We find that information on phage diversity enables identification of the bacteria that produced these phages, including pathovariant strains of *Escherichia coli*. Phage sequences can likewise be used to distinguish between closely related bacterial species such as *Staphylococcus aureus*, a frequent pathogen, and coagulase-negative Staphylococcus, a frequent contaminant. Phage cell-free DNA may have utility in studying bacterial infections.

Exploring the Sepsis-Delirium Connection Through Glycoproteomics

Steven Patrie, Ph.D., Northwestern University

This R21 project developed and validated a top-down proteomics (TDP) workflow for characterizing glycoproteoforms in sepsis-related biospecimens. The project addressed a critical gap in precision glycoproteomics by enabling direct mass spectrometric analysis of intact glycoproteins, capturing microheterogeneity beyond conventional glycan- or glycopeptide-based approaches. Our central hypothesis was that glycosylation-driven relationships in acute phase proteins (APPs) could be effectively represented using proteoform networks, providing novel insights into sepsis progression and its long-term effects, including cognitive impairment. Key accomplishments include the establishment of a reproducible pipeline for the comprehensive analysis of blood-based glycoproteoforms from small-volume plasma and serum samples. We developed optimized sample processing strategies, including protein depletion and lectin affinity enrichment, coupled with multidimensional separation techniques and TDP. A novel informatics framework, Proteoform Network Analysis (PNA), was implemented to automate the organization and quantification of glycosylation dynamics, facilitating robust site-independent glycosylation enzymology predictions. Additionally, we introduced machine learning tools for glycoform assignment and Predicted Pathway Optimization (PPO) for N-glycan mapping. The pipeline demonstrated the ability to systematically identify and quantify gps directly from patient plasma/serum, revealing persistent glycosylation changes even after APP concentration normalization. Notably, PNA analysis of Alpha-1-antichymotrypsin (AACT) glycosylation patterns distinguished patient subgroups based on sepsis onset and recovery timelines, underscoring the potential of glycoproteoforms as biomarkers for disease stratification. Advancements in data acquisition methods, including a data-independent acquisition (DIA) strategy incorporating in-source dissociation (ISD), further enhanced proteoform detection with controlled false discovery rates. Overall, these advancements position our workflow for broader clinical proteomics applications, offering a novel approach to investigations into the molecular heterogeneity of sepsis sub-phenotypes/endotypes.