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Second NIGMS Sepsis R21/R33 Biospecimens Investigators' Meeting

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Virtual Meeting

Meeting Report

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Executive Summary

The Sepsis Human Biospecimens Investigators' Meeting gathered National Institute of General Medical Sciences (NIGMS) grantees funded through the funding opportunity announcement (FOA) PAR-21-077. The purpose of this FOA was to support the efficient collection, biobanking, and sharing of biospecimens and associated clinical data from critically ill and sepsis patients for use in future mechanistic research. It grew from the need to accelerate fundamental discoveries that provide novel insights into the heterogeneity of the pathogenesis and resolution of human sepsis, which has not been achieved by studies that are overly reliant on murine models of sepsis.

Throughout the meeting, investigators presented ongoing research funded by the sepsis biospecimens program and discussed ideas, data, methods, and best practices for biorepository creation and use. The meeting was divided into two sessions. Session I focused on establishing scalable, multicenter biorepositories of septic patients, and Session II on developing or refining methodologies to embrace new opportunities and overcome challenges in sepsis research.

In Session I, Dr. Lorraine Ware presented the SCARAB R33 project focusing on collecting various types of samples and non-invasive sampling of distal airspaces in sepsis-induced ARDS patients using heat moisture exchanger (HME) filters, and addressing real-time phenotyping challenges. Implementing a waiver for timely consent and broad enrollment were key factors in their successful patient enrollment. Delayed syndrome adjudication, automated phenotyping algorithms, and a multi-organ approach aided in precise clinical phenotyping. The team has made significant progress in sharing their biorepository with other investigators.

Dr. Fran Balamuth presented her R33 project to build a multi-center pediatric sepsis biorepository and clinical database network on the basis of pre- and post-resuscitation samples from the same patient to reduce inter-individual heterogeneity. The team tested minimal blood volume for proteomics and immunophenotyping, delayed sample processing to accommodate low-resource sites, and harmonized a de-identified clinical data database network linked to biospecimens. The team showed promising results and is ready to prove the scalability to multiple sites.

Dr. Leopoldo Segal presented his R33 project focusing on high-resolution assays of paired lower airway and blood samples from COVID-19 patients. The team observed that local samples, particularly from the lower airways, provided more significant insights into poor outcomes than systemic samples. They also highlighted the importance of understanding site-specific differences in adaptive immune responses and transcriptomics. He revealed the need to adjust the complex metadata based on their various clinical stages when analyzing longitudinal data and the unique challenge of performing scRNAseq on lower airway samples.

Dr. Stephanie Prescot presented her R21 phase project on standardizing the collection, preservation, and storage of longitudinal biospecimens from very low birth weight infants in the neonatal intensive care unit (NICU). The project has successfully enrolled a significant number of patients and collected various types of samples through implementing productivity-boost strategies such as forming a dedicated committee of nurses, using visual aids to boost enrollment, and employing multiple communication methods. They met with challenges obtaining informed consent for day 0 samples, which highlighted the value of delayed consent. Initial characterization of the gut microbiome from stool samples has been successful, and collaboration with systems biologists and scaling up to additional hospitals is part of their future strategy.

Dr. Mihir Atreya presented one of his R21 projects focusing on the multiomics integration of epigenetics and transcriptomic data of sepsis samples using an existing pediatric biorepository, which offers appealing opportunities to identify meaningful targetable epigenetic pathways for the

treatment of sepsis, similar to advancements in cancer biology. The team successfully conducted extensive quality control on biospecimens, generating sizable, high-quality, temporally paired samples for RNA sequencing and DNA methylation analysis. Initial transcriptomic and methylation data reveal dynamic immune responses dominated by innate immune cells and point toward potential epigenomic drivers of multi-organ dysfunction. The team's experience highlighted the critical importance of high-quality, temporally matched samples, the need to adjust for batch effects and technical noise, and the limitations of bulk analyses in detecting cell- or tissue-specific signals. The team will incorporate single-cell analysis to address this gap in the future.

Dr. Annette Esper and Dr. Rishikesan Kamaleswaran presented their SIBER-AI R21 project using AI-driven alerts for early sepsis patient recruitment before the ICU, collecting early biospecimens including novel volatile organic compounds (VOCs), and devising novel consent materials. They deployed a sepsis screening algorithm, which successfully enriched sepsis patients and collected samples from unrecognized sepsis patients. The goal to collect respiratory VOC samples in an ambulance setting met with the challenge of time constraints, so they propose to use a newly developed rapid canister method going forward. Skin VOC sample collection in the ICU was successful and showed feasibility in capturing distinct VOC profiles. They also conducted patient and family interviews and formed a patient advisory panel to create a more patient-centered, straightforward consent process, incorporating feedback to develop a concise flyer and a video.

Session II started with Dr. Matt Foster presenting his R33 project to collect small, stable blood samples using MITRA tips and extract proteins for multiomic mass spectrometry analysis and deep sepsis phenotyping. They successfully quantified a large number of proteins, glyco- and phospho-peptides, and demonstrated strong correlations between their data and clinical assays. MITRA tips were shown to capture cellular components and reduce pre-analytical variability. Dr. Foster highlighted the potential for MITRA tip collection to be incorporated into routine clinical care and its value in longitudinal analysis that significantly reduces individual variability. Challenges remain in performing metabolomics on these samples.

Dr. Roby Bhattacharyya presented his Cryo-PRO R33 project, which was motivated by the need for high-resolution, single-cell multiomic data to understand the heterogeneity of sepsis. Traditional methods for single-cell sequencing are laborious and costly, but Cryo-PRO streamlines sample processing by adding a cryoprotection step and isolation of peripheral blood mononuclear cells (PBMCs) later at a central lab, making it feasible to embed single-cell studies into clinical workflows. The team optimized post-thawing procedures to ensure high-quality scRNAseq data. Comparative studies showed that Cryo-PRO generated data of similar quality to traditional Ficoll methods, with indistinguishable cell viability, gene recovery, and biological metrics from scRNAseq studies. The R33 project is integrated into the APS consortium as an ancillary study to test its capacity to add value to existing large-scale observational studies, making it the largest single-cell study in sepsis to date.

Drs. Cristina Furdui and D. Clark Files presented their R33 project developing a redox-trapping cocktail (RMX), based on the critical role of redox metabolism in sepsis progression and the need for preserving the redox state of blood samples to identify reliable biomarkers, which was not done in traditional methods for blood collection. The RMX cocktail was formulated to prevent the formation of damaging reactive oxygen species, stop the propagation of radical reactions, and protect proteins from oxidative damage. The group showed that RMX effectively improved the quality of blood samples during freeze-thaw cycles and long-term storage, while it did not interfere with common clinical lab assays, except for some ion measurements. It significantly improved the stability of fragile biological biomarkers, such as VEGF and lactic acid. The team is scaling up the test in a multi-site observational trial and exploring if redox preservation leads to the discovery of novel sepsis biomarkers.

Dr. Eric Schmidt presented his R21 project aiming to optimize the DMMB assay to measure endothelial glycocalyx degradation as a point-of-care assay for sepsis management. This builds on the group's recent discovery using septic samples from the CLOVERS clinical trial, showing that circulating heparan sulfate (HS) is a strong prognostic biomarker for sepsis mortality, while chondroitin sulfate (CS) predicted differential responses to fluid resuscitation strategies. Initial results showed that the DMMB assay could quantify glycosaminoglycans in plasma and urine samples, potentially offering a rapid, inexpensive point-of-care test for sepsis management. Though some variability was noted, the team is refining the assay by adding a protease digestion step to reduce background. In the future, they will perform the DMMB assay at different stages of sepsis and correlate with endothelial injury markers to see if it can offer granularity for sepsis severity.

Dr. Basilia Zingarelli presented her R21 project on testing the utility of sEVs isolated from an existing biobank in sepsis research. The team isolated sEVs from plasma and serum samples of septic patients and controls, finding that PS-affinity extraction provided higher purity and yield than ultracentrifugation. They tested the immunomodulatory properties of sEVs and found that sEVs from sepsis patients influenced NF-κB responses differently than those from controls. In RNA analyses, they identified specific RNA biotypes associated with clinical characteristics such as organ failure. Moving forward, the team plans to validate these findings in a prospective study and explore the use of sEVs as diagnostic or prognostic biomarkers in sepsis.

Dr. Mihir Atreya and Dr. Andrew Lautz presented their R21 project on establishing a biobank of patient-specific induced pluripotent stem cells (iPSCs) for pediatric sepsis research. The team reprograms somatic cells into iPSCs and differentiates iPSCs into different cell types. They successfully generated iPSCs from a two-step cell enrichment protocol. Dr. Lautz showed that serum from sepsis-associated cardiovascular dysfunction (SAMD) patients reduced contractility in sepsis patient-specific iPSCs but not in healthy iPSCs, highlighting the importance of genetic components in sepsis-related cardiomyopathy. In addition, higher-risk serum induced inflammatory gene expression in iPSC-derived monocytes, as well as increased glycan and angiotensin signaling and stress responses in iPSC-derived endothelial cells. Future plans include expanding the iPSC biobank to capture more genetic diversity, comparing responses to primary cell types, studying epigenomic factors, and establishing organ-specific organoid models of sepsis dysfunction.

Each of the two sessions was followed by a panel discussion. Summary of key points and expert advice to the community of sepsis biobanking is as follows:

1. **Novel Consent Process**: Novel consent approaches are necessary for clinical studies on critical illnesses. Initial waiver or delayed consent is common and helps to avoid inclusion bias and sample missingness. A potential challenge is different levels of IRB acceptance at various institutions. The discussion emphasized the importance of publishing novel consent processes and experiences to convince IRB committees of their validity. It is of interest to compare different consent models in the U.S. and abroad to provide insights into the efficacy and acceptance of these models. Community consultation and pre-enrollment conversations are often crucial for successful informed consent. The importance of effective consent materials and the operation team is also highlighted.
2. **Value of Remnant Samples**: Communication with the local IRB is recommended to find out what remnant samples can be collected without informed consent. Storage duration of these samples depends on the clinical lab's protocols, while in some cases, investigators can obtain them more freshly when applying proactive logistics. Residual samples are suitable for preliminary data and proof-of-concept studies, as well as some stable assays (e.g., proteomics), but they might not be ideal for validation and sensitive assays (e.g., metabolomics).

3. **Strategic Decision-Making in Biobanking**: Investigators touched on a few aspects of biobanking, such as aliquoting vs. utilization, purpose-driven vs. broad sampling, and intentional distribution. Novel and economic methods of sample storage were called for to ease the economic burden. Scientific question-based sample collection (e.g., the APS consortium) plus oversampling and broad sampling could achieve a healthy balance. Long-term storage has value for certain samples, such as iPSCs and PBMCs, while instant processing and data sharing may be better for other samples. For goal-oriented sample collections, assays are often planned out at the time of sampling. An interesting concept of treating a biobank as a "living organism" was brought up, balancing the need to grow and to distribute the collected samples. Future utilization should be considered when designing informed consent. Sharing successes and challenges via publications in this evolving process is crucial for advancing the field. Promoting awareness of available samples was also thought to be important (through NIH NOFOs or collaborations among investigators).

4. **Sample collection from clinical trials (CT)**: Investigators highlighted the value of samples collected during CTs in understanding patient responses to treatments, which had led to seminal discoveries in the critical illness space. This seems to be a current gap since NIH support in efficacy CTs has been declining, while industry-sponsored trials present challenges in ownership. Nevertheless, NIH still supports mechanistic CTs. The value of simulated clinical trials was brought up, which can potentially offer insights to support the feasibility of CTs based on causal inference. The caution is that simulation trials in critical care are mathematically complex due to the dynamic nature of the disease, though adding continuous data could mitigate such biases.

5. **Control samples:** The discussion on control samples emphasized the importance and challenges of collecting these samples to answer key biological questions. Existing literature showed that there are early transcriptional signatures in host response that can dictate the chance of developing sepsis in both adults and neonates. One difficulty in collecting early pre-disease samples is predicting which patients would develop sepsis, given the low incidence (<1% among patients in the Emergency Department/ED), while common clinical indicators (e.g., positive culture) are not reliable predictors for sepsis. Electronic health record (HER) Sepsis alert may not be ready for patient diagnosis, but it is very useful in enriching sepsis patients for research. The potential value of collecting healthy samples during newborn screening offers an excellent opportunity for this purpose. Oversampling in small volumes (such as dried blood spots) is another strategy to capture critical early samples. Another important group to capture is patients who recover from sepsis without progressing to severe illness.

6. **Buy-in of novel samples and methods**: Discussion focused on the applicability and integration of novel sample collection and processing methods into large trials. Investigators are interested in methods to enhance efficiencies in multi-site trials, and increasing awareness is the key. It is also important to educate the user about the added value and caveats of the new technology, so further validation and broad testing under various scenarios are critical. High-value method (e.g., Cryo-PRO offers scRNAseq capacity) has good buy-in by the users. Investigators shared their thoughts on novel technologies in Session II, including Cryo-PRO, redox cocktail, DMMB assay, and HME filters. *De novo* designs often meet with unanticipated problems, while new methods designed to utilize what is already in the clinical workflow work well. In addition, some methods are ready for scaling, others are not yet mature. Investigators emphasized the importance of both mature and early-stage methods for maintaining continuity in the biobanking innovation pipeline. Development of these should not be separated from the research question. New technologies should be compared side-by-side with standard methods in large trials for final validation and wider adoption.

7. **Sharing practice to improve reproducibility**: Internal technical normalization and paired samples are important to ensure data quality and reduce individual variability. Sample stratification helps to remove known confounders for certain analyses (e.g., the effect of sex on epigenetics).

Investigators pointed out the benefits of accumulating multiple datasets from large cohorts as well as diversifying the dimension of analysis for samples from the same patients. This could improve reproducibility by reducing possible biases of certain datasets. Creating reference materials centrally is suggested, and the National Cancer Institute Early Detection Research Network biobank program was brought up as a good example of doing so. Coming up with a common set of quality matrices for certain sample types should be useful (e.g., acceptable volume for bronchoalveolar-lavage fluid/BALF). Investigators further emphasized the importance of reference and standards and suggested that some human samples may be reserved to bridge the adoption of new technologies, i.e., testing a range of new methods and creating reference results for multiple projects in the same set of samples. It is conceivable that a U54 funding mechanism might help achieve these goals.

8. **Hurdles of sharing:** Investigators discussed hurdles around sharing biospecimens and data, including institutional and contractual obstacles, the need to balance high standards with practicality, and the challenge of managing resources, often with limited funding. Participants noted the value of centralized or federated repositories, such as NIH's BioLINCC, but also pointed out difficulties in timely accessing and understanding available resources in these central repositories. There was a consensus that the community must collaborate on practical mechanisms and infrastructure to facilitate efficient and reproducible sharing and maximize the impact of collected samples and data.

Welcome, Introductions, and Opening Remarks

Jon Lorsch, Ph.D., Director, NIGMS
Rochelle M. Long, Ph.D., Division Director, NIGMS
Xiaoli Zhao, Ph.D., Program Director, meeting organizer, NIGMS
Chien-Chung Chao, Ph.D. Program Director, meeting organizer, NIGMS

Dr. Xiaoli Zhao, Program Director, National Institute of General Medical Sciences (NIGMS), opened the meeting and welcomed those on the call to the second NIGMS Sepsis Human Biospecimens Investigators' Meeting. She introduced Dr. Jon Lorsch, Director, NIGMS.

Dr. Lorsch opened the meeting by expressing excitement about gathering virtually to hear the latest results from the project, which was initiated several years ago to support the APS consortium by developing new methods and exploring the utility of existing biospecimen collections that might be underused.

He reflected on the R21/R33 funding mechanism, explaining that it was intentionally designed to foster high-risk, high-reward projects, and noted that about half of the funded projects transitioned from R21 to R33, which matched their expectations and indicated they had chosen appropriately ambitious projects. Dr. Lorsch emphasized that both the projects that advanced and those that did not are considered successes, as the goal was to encourage risk-taking and innovation.

Looking ahead, he suggested the field would benefit from the involvement of more biochemists, given that sepsis is fundamentally a biochemical issue, and underscored the importance of expertise in pathways, interactions, and kinetics. He also mentioned that, in addition to biochemists, the field needs more systems biologists and data scientists, as well as greater integration of AI and data-driven approaches, to accelerate progress in sepsis research.

Following Dr. Lorsch, Dr. Rochelle Long began her opening remarks by expressing appreciation for the hard work of the meeting's organizers and highlighting Dr. Zhao's efforts in establishing and overseeing the program. She reiterated that the goal of the initiative is to support the efficient collection, banking, and sharing of biospecimens linked with clinical data from critically ill patients, all to advance mechanistic research into sepsis and understand its biochemical underpinnings.

Dr. Long then outlined that the R21 phase focused on testing new biospecimen acquisition methods, assessing the quality of existing collections, and developing useful assays, while the R33 phase now involves scaling up these activities to generate substantial datasets for hypothesis-driven research on sepsis prediction, development, and resolution.

She thanked investigators for their commitment to ongoing collaboration through regular meetings and emphasized the shared goal of accelerating discoveries to better understand and ultimately treat sepsis, ending with further gratitude to both the attendees and the organizers.

Dr. Zhao acknowledged the inspiring vision outlined by Drs. Lorsch and Long, and emphasized the importance of strong leadership support for the program's success. She warmly welcomed all attendees, including sepsis biospecimen principal investigators (PIs), the pneumonia, sepsis, and acute respiratory distress syndrome (APS) consortium investigators, and the National Heart, Lung, and Blood Institute (NHLBI) colleagues, highlighting the high level of enthusiasm and collaboration that drives the program forward.

Reflecting on the program's progress since its inception over two years ago, Dr. Zhao noted the significant achievements since the first investigator meeting, such as numerous publications, the

development of sharable biorepositories, and innovative methodologies. She celebrated the successful integration of the sepsis biospecimens program with the APS consortium, with some projects now serving as ancillary studies. Dr. Zhao concluded by underscoring the meeting's focus on science and invited her co-organizer, Dr. Chien-Chung Chao, to speak.

Dr. Chao expressed his gratitude to everyone who contributed behind the scenes to make this meeting possible. He extended special thanks to Nancy Hernandez and Sushma Jammula for their assistance with the meeting registration website, the communication and public liaison team for assembling the abstract book that was distributed to attendees in advance, and Tony Baum's administration team for providing technical support before and during the meeting. He concluded by saying he looks forward to the upcoming research updates and insightful panel discussions and thanked everyone for their participation.

Objectives and Charge to Presenters and Participants

Meeting Co-Chairs:
Lorraine Ware, M.D., Vanderbilt University Medical Center
Michael Filbin, M.D., Harvard Medical School and Massachusetts General Hospital

Dr. Chao introduced the meeting's co-chairs, Dr. Ware, Professor of Medicine from Vanderbilt University Medical Center, and Dr. Michael Filbin, Associate Professor from the Department of Emergency Medicine, Harvard Medical School and Massachusetts General Hospital.

Dr. Ware began by expressing her gratitude to the organizers and her enthusiasm for the scientific discussions ahead, particularly advances in human sepsis research and innovative biobanking methods. She emphasized the critical importance of improving clinical research methods to better understand sepsis pathogenesis and ultimately develop targeted therapies. Representing both her NIGMS program and a clinical center-specific project within the APS consortium, Dr. Ware highlighted her interest in fostering greater synergy between the two programs to advance biobanking to the cutting edge.

Dr. Filbin followed by thanking NIGMS for funding this important initiative, which has created valuable opportunities for collaboration and idea-sharing among sepsis researchers. He noted the program's role in fostering interdisciplinary partnerships across specialties such as emergency medicine, critical care, infectious diseases, and immunology. Reflecting on the first investigator meeting, Dr. Filbin praised the comprehensive meeting report for the first investigator meeting, especially the executive summary, for effectively capturing key discussion points and guiding ongoing collaborative efforts in sepsis research. He emphasized the following key concepts introduced in the first meeting report and encouraged participants to keep these ideas in mind during the presentations at this second meeting. By doing so, he hoped the discussions would build on previous insights and help advance the collective progress of the group.

- Utilization of remnant samples
- Adoption of emerging technologies
- Leveraging state-of-the-art methods to enable collection from small sample volumes
- Clearly defining the ontology and protocols for biorepository sample collection
- Establishing standardized processing workflows as a foundational step
- Incorporation of comprehensive and thoughtful clinical annotation
- Setting reference standards for ground truth
- Addressing pre-analytic variability in patient samples to ensure accurate biological insights
- Implementation of automated EHR screening and retrospective syndrome adjudication

- Appropriate control groups
- Balancing broad versus focused inclusion criteria
- Collection of paired samples before and after treatment
- Emphasizing the importance of longitudinal and time-zero sampling
- Exploring different consenting mechanisms
- Weighing the philosophy of open-ended discovery against hypothesis-driven research
- Utilizing a centralized and structured database for depositing datasets from diverse sources, supported by high-capacity computational tools

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

Session Co-Chairs: *Fran Balamuth, M.D., Ph.D., The Children's Hospital of Philadelphia; Leopoldo N. Segal, M.D., M.S., New York University School of Medicine*

Dr. Ware introduced the chairs for the first session this morning: Dr. Fran Balamuth from the Children's Hospital of Philadelphia and Dr. Leopoldo Segal from NYU. Drs. Segal and Balamuth introduced the speakers and moderated the Q&A following each presentation.

The Sepsis ClinicAl Resource And Biorepository (SCARAB) Project

Lorraine Ware, M.D., Vanderbilt University Medical Center

Dr. Ware presented their R33 biorepository project, co-led with Dr. Julie Bastarache, designed to address several major challenges in clinical sepsis research. The first challenge is the difficulty in non-invasively sampling the distal airspace in patients with sepsis-induced ARDS or those mechanically ventilated without ARDS. Traditional bronchoscopic or non-bronchoscopic BAL procedures are invasive and often not feasible in all patients. To overcome this, the team developed a novel, non-invasive method that uses heat moisture exchanger (HME) filters from ventilator circuits to collect distal airspace fluid, enabling regular sampling for biomarker and mechanistic studies.

The second challenge is obtaining timely informed consent from critically ill, sedated, or comatose patients, who often lack available surrogates early in their ICU stay. The project addresses this by using a waiver of timely informed consent when necessary, allowing for early patient enrollment and biospecimen collection, with consent pursued as soon as possible thereafter.

The third challenge centers around real-time phenotyping for sepsis and ARDS, as key clinical data are often unavailable at enrollment. To address this, the team enrolls nearly all critically ill patients who may have sepsis, then applies deep phenotyping retrospectively as more clinical data become available, improving accuracy in syndrome classification.

Additionally, the project confronts the labor-intensive nature of clinical data collection by developing phenotyping algorithms that leverage EHR data to identify lung injury and multiple organ dysfunctions in a multitude of sepsis patients more efficiently. The team focused on multiple organs rather than just a siloed approach to individual organ dysfunction, which fits very well with the NIGMS mission not to be organ-centric in approaches to study sepsis. They also aim to create infrastructure and workflow to make the collected samples and data more accessible to other investigators.

The project's key achievement is the innovative use of HME filters from ventilator circuits to non-invasively collect fluid that accurately reflects the biology of the distal airspace in critically ill

patients, as validated by comparable levels of biomarkers like IL1β and RAGE to those found in pulmonary edema fluid. In the R33 phase, the team established a two-center biorepository enrolling over 420 adult and pediatric patients, including underserved populations, across a broad spectrum of critical illness, with serial biospecimen collection (blood, DNA, RNA, urine, and HME fluid) at multiple time points.

The project also developed and validated automated EHR-based phenotyping algorithms, including a decision tree classifier for ARDS and a sepsis classifier, enabling efficient and accurate identification of cases and controls for research.

The SCARAB program also supports a range of innovative projects leveraging its biorepository, overseen by an external advisory board to facilitate sharing. Avery Bogart investigated epithelial glycocalyx shedding in ARDS, finding that Syndecan-1 levels in HME filter fluid were significantly higher in ARDS patients than in those with cardiogenic pulmonary edema, linking glycocalyx injury to ARDS severity and mortality. Matt Stier used single-cell RNA sequencing and SCENITH metabolic profiling on immune cells from SCARAB patients, discovering that Treg cells in sepsis acquire increased glycolytic capacity and express more suppressive markers, with these changes tied to illness severity and driven by altered Kynurenine metabolism. Alicia Rizzo's research revealed that males experience greater epithelial glycocalyx shedding than females in ARDS, highlighting sex differences in sepsis-related lung injury. Additionally, X-zavyer Smith conducted a study using skin imaging to examine leukocyte-endothelial interactions in critical illness, further expanding the program's research impact.

Key lessons learned include the value of early enrollment, broad inclusion, and delayed phenotyping. The team continues to refine their phenotyping algorithms, facilitate data sharing, and expand collaborations, particularly with the APS consortium. A key challenge in ARDS research is how to integrate diverse data types. For example, imaging results require natural language processing (NLP) to extract relevant information from text. Her team is actively refining NLP algorithms to improve this aspect of their models. Dr. Ware concluded by emphasizing that their biorepository samples are available for collaborative research, furthering the understanding of sepsis pathogenesis and supporting the development of targeted therapies.

In the Q&A session, Dr. Segal raised the challenge of handling complex clinical metadata in research, specifically whether to treat each variable independently or to group related variables due to potential collinearity (e.g., multiple indicators for renal function). Dr. Ware responded that having a manually phenotyped cohort linked to EHR data allowed her team to test different ways of weighting, grouping, and modeling variables in a validated population.

Dr. Zhao asked about the technical expertise needed for physicians or basic scientists to engage in data science and systems biology. Dr. Ware explained that her group relies heavily on biomedical informatics colleagues for EHR data extraction, NLP, and advanced statistical modeling. As they move from simple classifiers to more complex machine learning models, significant statistical expertise is needed. For specialized analyses like scRNAseq and immunometabolism, collaboration with experts in those fields (e.g., Dr. Kimryn Rathmell for SCENITH analysis) is essential. Overall, successful research in this area requires multidisciplinary collaboration.

Developing a Scalable, Multicenter Pediatric Sepsis Biorepository and Clinical Database

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

Dr. Balamuth opened her presentation by expressing gratitude to NIGMS for supporting a program that brings together investigators from various clinical backgrounds and includes sepsis patients

across a broad age range. She highlighted the program's commitment to pediatric research, including a neonatal project that she is particularly eager to learn more about, given the ongoing debates regarding the similarities and differences in pathophysiology between neonates and other pediatric sepsis patients. Dr. Balamuth introduced herself and her MPI, Dr. Nelson Sanchez-Pinto, noting their respective expertise in pediatric emergency medicine and pediatric critical care.

The overarching goal of their project is to address the challenge of studying sepsis phenotypes at scale in children, recognizing the disease's inherent heterogeneity. The team aims to systematically collect and analyze biological samples and clinical data at three distinct phases of care: before resuscitation, during resuscitation, and after resuscitation. Their vision is to develop tests that can identify high-risk pediatric sepsis patients early in the emergency department, enabling clinicians to stratify patients into subphenotype groups and ultimately deliver more targeted therapies. To achieve this, the project is designed to establish a scalable multicenter biorepository that is both low-cost and feasible for implementation in resource-limited settings, including non-pediatric centers, nonacademic hospitals, and low- and middle-income countries. The team also seeks to optimize the blood volume required for various assays, perform immune phenotyping and immunometabolic analysis using the SCENITH platform, and capture the clinical heterogeneity of sepsis through high-dimensional EHR data.

During the initial R21 phase, the team concentrated on several foundational tasks. They rigorously tested the minimal blood volume necessary for three different types of assays, i.e., proteomics, immune phenotyping, and immunometabolic analysis, since drawing large volumes of blood from children is not always practical. In addition, they investigated how long blood samples could remain at room temperature before processing, which is a critical consideration for sites with limited resources that need to ship samples to centralized analytic centers. The team also developed a parallel clinical data repository that allows for the sharing of de-identified patient data while maintaining the crucial linkage to biospecimen metadata.

Operationally, the team established a workflow for identifying eligible patients using a sepsis trigger embedded in the EHR at the time of emergency department presentation. This trigger not only facilitates real-time sample collection but also enables retrospective identification of patient records for data extraction. A waiver of informed consent was proven highly effective for the team to collect an initial blood sample early in the emergency department, with a 92% success rate for the first time point (T0). However, many of these patients were co-enrolled in an ongoing clinical trial that was operating under informed consent. For subsequent samples, prospective consent was required, resulting in a 60% consent rate, and the team successfully collected second and third timepoint samples from approximately 50% and 25% of eligible patients, respectively. Samples were discarded if the patient declined the informed consent. There is also a broader waiver of consent for all the patients who have sepsis triggers to get their EHR data, even if they declined biospecimen collection.

Through these efforts, the team demonstrated that a blood volume as low as 2 ml is sufficient for the planned assays. Proteomic analysis was best performed on fresh plasma samples. PBMC immune phenotypes were minimally affected by this overnight processing delay of 12-24 h, but not at longer time intervals to blood processing (e.g., overnight shipping). Flow cytometry immunophenotyping results indicate the loss of peripheral CD8+ T cells in high-severity patients.

The team faced a workflow challenge of promptly isolating fresh plasma while isolating PBMCs later. This was addressed by either having clinical labs perform plasma isolation around the clock at one site or by research teams handling it for most of the day at other sites. Both ensure high-quality samples that were then de-identified and matched with clinical data.

Regarding the clinical database, in the R21 phase, data pipelines were established and quality-checked locally, with harmonization using the OMOP model and standardized coding systems such as ICD-10 and LOINC. In the R33 phase, the team has expanded the clinical database pipeline by using the PEDScreen decision tool to extract sepsis data from the EHRs at CHOP and Lurie, de-identifying subjects, and securely transferring and harmonizing the data at Lurie with biospecimen metadata. This process is now being implemented at the Colorado site, where PBMC collection and data harmonization are underway. So far, about 2,000 pediatric patients have had sepsis-flagged visits, with biospecimens collected from 42. These patients tend to be sicker based on organ dysfunction and treatment needs. In the next step, the team will use this harmonized dataset to classify immune phenotypes. The team is also developing a cloud-based application for collaborators to access data and samples and has presented early findings at the 2024 Critical Care Congress.

In the Q&A session, Dr. Chris Seymour asked about sample storage temperatures, and Dr. Balamuth explained that their samples were kept at room temperature, with holding times tested using whole blood. Dr. Foster asked about validating Olink assays with UK biobank data; Dr. Balamuth said they have no current plans but are open to discussion. Chat comments noted that Olink and mass spectrometry correlate better with ELISA than SomaScan, and that mass spectrometry offers unbiased peptide sequencing, while SomaScan detects a different protein set.

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

Dr. Segal's lab, supported by multiple major funding agencies, shifted its focus to COVID-19 at the start of the pandemic, leveraging expertise in microbial-host interactions and omics data analysis. This current work centers on understanding whether the poor outcomes in critically ill COVID-19 patients were due to secondary infection, viral toxicity, or the exuberant inflammation. Using single-cell RNA sequencing (scRNAseq) and other omics approaches on paired lower airway and blood samples, the group will identify key cell types and molecular pathways associated with mortality and poor clinical outcomes, distinguishing local (airway) versus systemic (blood) responses, and building a high-quality, longitudinal cohort for ongoing and future studies. He applauds NIGMS's vision for funding quality control (QC) and troubleshooting projects for processing samples, which is an important gap in the field.

To date, Dr. Segal's group has prospectively enrolled over 500 intubated, critically ill patients, focusing on the collection of longitudinal samples and clinical metadata. Later enrollment included sepsis due to viruses other than SARS-CoV-2, which opened a very interesting new venue of investigation. Early findings from his group indicated that patients with poor outcomes exhibited higher viral loads but blunted adaptive immune responses, especially in the lower airways. Bulk RNA sequencing of blood samples revealed that key inflammatory and metabolic pathways failed to upregulate in patients who died or required prolonged intubation, while many critical pathways were downregulated. The team stratified over 200 patients into discovery and validation cohorts for transcriptomic studies, consistently identifying neutrophil dysfunction, interferon signaling, and inflammatory pathways (such as IL-6 and TNF) as associated with mortality. Notably, there was only partial overlap between blood and airway transcriptomic signatures, with more distinct signals in the lower airway, particularly in the second week after intubation. T cell function was found to be downregulated in the blood but upregulated in the lower airways, which is associated with poor outcomes and mortality. These findings underscored the value of local airway sampling and longitudinal analysis.

The study faced several operational and analytical challenges, particularly for the scRNAseq assay. Patients were grouped into rapid progressors, slow progressors, poor responders, and good responders to easier comparison of a very complex cohort, and they also selected a case control subgroup for the analysis that was matched for clinical aspects and confounders, which contained almost half who had poor outcomes as defined by mortality. Time zero was difficult to standardize, as patients reached intubation at different points in their disease course, making it critical to carefully interpret sample timing in relation to disease progression. Cryopreserved lower airway samples were challenging due to differential cell sensitivity to thawing and processing. Cell hashing worked for PBMCs but failed for most lower airway samples. The annotation of single-cell data for lower airway cells was particularly complicated by the lack of suitable reference datasets. In addition, collecting longitudinal samples and performing analysis longitudinally, as well as integrating data with other omic datasets such as metabolomic data, present a major analytical challenge. As there is no roadmap for doing this, there is an opportunity for innovation, but one has to exercise caution and form collaboration with bioinformatics groups.

Dr. Segal's group established a large, well-characterized cohort of critically ill COVID-19 patients, with robust longitudinal sampling and metadata collection. Their integrated omics approach revealed that poor clinical outcomes are linked to both systemic and local immune dysregulation, with lower airway samples providing unique and more granular insights compared to blood alone. Analytical challenges, particularly in single-cell work and sample annotation, were partially overcome through rigorous quality control and method development. The team is expanding its investigation into other viral causes of sepsis, aiming to determine whether observed immune mechanisms are unique to COVID-19 or shared across respiratory viruses. The project's success to date rests on careful sample handling, detailed clinical linkage, and commitment to methodological rigor and collaboration, especially given the complexity and heterogeneity of critical illness.

In the Q&A session, Dr. Zhao asked whether centering clinical metadata around intubation reduced heterogeneity and what biological mechanisms could be inferred. Dr. Segal responded that while such centering helps normalize data and reduces subtle differences between various clinical settings (i.e., ED, ICU, post-ICU), patients do not follow a uniform disease trajectory, and careful adjustment for confounders remains essential. Dr. Kamaleswaran questioned how differences between BAL and blood samples might reflect primary infection sites and whether conserved signatures might be found to indicate systemic inflammation despite the primary infections. Dr. Segal highlighted that the pandemic provided a unique opportunity to study a large cohort with a single viral cause, but as the cohort expands to other viruses and types of sepsis, differences in underlying mechanisms will become increasingly apparent. He emphasized the need for large, collaborative consortia containing different primary diseases to address such complex questions.

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

Dr. Prescott's R21 project aims to establish rigorous and reliable protocols for sample collection, preservation, and storage in the NICU, focusing on very low birth weight infants born under 1,500 grams or before 33 weeks' gestation. The team seeks to capture blood, stool, saliva, urine, skin swabs, and feeding samples alongside detailed demographic and clinical data at critical time points before, during, and after suspected sepsis. In the short term, the objective is to determine the optimal collection methods and storage conditions to ensure sample integrity. The long-term goal is to leverage these standardized biobanking practices in the R33 phase, incorporating systems biology

analyses in order to develop predictive models and point-of-care testing to classify and guide treatment of neonatal sepsis.

To date, the team has successfully enrolled 84 infants, including 44 growing preemies, 20 culture-negative sepsis cases, and 20 culture-positive sepsis cases, nearly meeting the enrollment target of 88 participants across Tampa General Hospital and INOVA Children's Hospital. Although day-of-life T0 sampling proved challenging due to parental stress, obtaining consent by day 3 became routine, enabling collection at days 3 and 7 and then weekly up to seven weeks of life. This effort has yielded over 350 sample time points, while later time points from some 32 weekers were missing because they were discharged before reaching 7 weeks. Dr. Prescott noted the vital importance of pre-sepsis sample acquisition for downstream systems-biology analyses.

The study encountered contractual delays, single-IRB complexities, and RedCap access hurdles, especially at non-research-experienced sites. Competing studies and low consent rates at the initial teaching hospital slowed enrollment, while challenges were later mitigated by moving to a nonteaching facility that now enrolls three to five infants weekly. Communication gaps were addressed by forming a dedicated nursing committee that conducts daily rounds to identify eligible patients, oversee sample collection, and ensure proper storage. The team developed a comprehensive CHEATSHEET and inventory system to guide nurses through sample type, collection tubes, and processing workflows. They learned that approaching families between 24 hours and three days postpartum maximizes consent rates, aided by parent information letters, and "super baby" room signage that both informs staff and engages families. Embedding sample reminders within Epic order sets and chart headers, coupled with daily interdisciplinary rounds to troubleshoot collection barriers, has proven essential for consistent protocol adherence.

The team collected longitudinal whole blood samples from heel sticks in very small quantities (250–500 µl) for bulk RNAseq. There were some variations in obtaining the first sample before or after the antibiotics, which has to be administered within 1h of sepsis diagnosis. The team evaluated various sample collection and preservation methods for microbiome and biomarker analysis in neonates, comparing rectal swabs versus stool samples (stored at -80°C, room temperature in 95% ethanol, or lysis buffer) and different salivary collection techniques (cotton swabs, flox swabs, and passive drool) for protein analysis, since it is difficult to collect passive drool from neonates despite it being the gold standard.

Microbial population analysis revealed no significant differences between stool and rectal swab samples or among different storage conditions, though some species-level differences were detected, particularly in skin- and mucous membrane-associated microbes present in rectal swabs, which may be relevant for studying necrotizing enterocolitis. As a result, the team decided to retain both stool and rectal swab samples under optimized storage conditions. LEfSe analysis confirmed minimal taxonomic differences across stool sample methods, with only minor variations in rectal swabs. For saliva, DNA and protein yields were similar across collection methods, except that fresh processing provided superior DNA quality, though frozen samples remained acceptable; cortisol measurements showed no significant difference in protein levels between passive drool and Weck-Cel flox swabs. Finally, bulk RNA extraction from frozen whole blood produced adequate RNA quantities, though initial quality was suboptimal until the optimal preservation buffer was identified.

LEfSe analyses revealed no significant differences in microbial population except for some minor species variations between frozen stool, ethanol-preserved stool at room temperature, and rectal swabs. There are minor differences in skin and mucous membrane-associated microbes in rectal swabs, warranting their continued use for investigating necrotizing enterocolitis. Saliva collection methods produced comparable DNA and protein yields, with fresh processing delivering superior

DNA quality but frozen samples remaining within acceptable parameters. Bulk RNA extraction from small heel-stick blood volumes yielded a sufficient quantity despite lower quality, prompting the identification of an optimal preservation buffer.

Going forward, the team will continue collecting samples. Dr. Prescott noted the strength of their repository, which includes patients with different clinical trajectories, such as those who developed sepsis after initially negative cultures and patients with pre-septic samples. They aim to maximize sample quality using the optimized collection and preservation methods established in the R21 phase. The multidisciplinary team will integrate microbiome, transcriptome, and proteome data to identify important biomarkers, with the ultimate goal of developing point-of-care diagnostic tools for neonatal sepsis.

In the Q&A, Dr. Matt Foster asked about the possibility of using remnant samples from day zero or cord blood. Dr. Prescott confirmed that cord blood collection is possible but requires pre-delivery consent, although a waiver of informed consent could be considered. Dr. Foster also suggested newborn screening as a source of samples, but Dr. Prescott noted that while this is routine for term newborns, it is not typically performed in preterm babies.

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

Mihir R. Atreya, M.D., M.P.H., Cincinnati Children's Hospital Medical Center and University of Cincinnati

Dr. Atreya, representing Drs. Nelson-Sanchez Pinto and Rishi Kamaleswaran introduced their R21 study aimed at advancing the mechanistic understanding of pediatric septic shock subtypes by leveraging an existing biorepository. He began by contrasting sepsis with Mendelian diseases such as cystic fibrosis and sickle cell anemia, which are driven by rare, high-penetrance genetic variants. Sepsis arises from numerous common variants with low penetrance, making genome-wide association studies (GWAS) less effective in identifying consistent mechanistic targets. Even when GWAS does yield insights, it often lacks broad applicability across the diverse patient population. The host response in sepsis is highly dynamic and redundant, with nearly 25% of the genome showing differential expression within hours of onset. This complexity is compounded by factors such as host-pathogen interactions, making it difficult to isolate upstream, causal mechanisms.

To address this challenge, the study emphasizes the need for a multi-layered molecular approach. The central dogma of molecular biology, DNA transcribed into RNA and translated into protein, becomes increasingly intricate in critically ill patients, and understanding this complexity requires integrating data from various “omics” layers, including the genome, transcriptome, proteome, and metabolome. These layers provide orthogonal evidence that, when combined, can yield deeper insights into the biology of critical illness. The team’s specific focus is on epigenomic modifications in pediatric septic shock, an underexplored area. While previous research has examined DNA methylation, other epigenetic mechanisms such as histone modifications and long non-coding RNAs may also influence gene expression and host response. The study aims to uncover these epigenomic shifts regulating sepsis progression and whether they could serve as viable therapeutic targets.

The biorepository they use is a two-decade, multi-center observational cohort of pediatric septic shock patients, originally based on 2005 consensus criteria and now also using updated Phoenix score assessments. It collects whole blood for DNA, RNA, serum, and plasma on days 1 and 3, and recently expanded its age range to include patients up to 18 years old. The R21 phase focused on ensuring biospecimen quality and generating preliminary DNA methylation and transcriptome data to explore epigenomic regulation of gene expression. The R33 phase will scale up these analyses,

aiming to define and validate multiomic sepsis endotypes, and develop an EHR-based clinical classifier to predict these biologically defined subtypes.

For aim 1, the team conducted rigorous multi-level internal and external QC on biospecimens in their biorepository to ensure suitability for high-throughput multiomic analyses. For DNA, the team will run Illumina methylationEPIC 850K microarrays, and for RNA, they will perform bulk mRNA sequencing at a read depth of 40 million reads per 150 bp. QC was completed on 851 RNA and 648 DNA samples from the biobank; 719 RNA and 542 DNA samples met the established quality metrics, surpassing R21 phase milestones. Transcriptomic data were generated for 202 patients at both day 1 and day 3, with an additional 215 high-quality, temporarily paired RNA samples available for sequencing. DNA methylation profiling was performed for 106 day 1 samples, and high-quality DNA is available for 432 additional patients for future R33 phase analyses.

Dr. Atreya showed a heatmap indicating that the majority of the existing biospecimens met the stringent QC metrics established. In total, the team generated 417 matched or temporarily paired pediatric septic shock biospecimens, exceeding the R21 phase milestones. Their protocol paper, published in The Lancet Ebiomedicine, described a methodology focused on organ dysfunction trajectories rather than mortality signatures. Using supervised machine learning, the team reduced the number of genes needed to predict persistent multi-organ dysfunction in patients. Two additional papers are in preparation describing temporal transcriptomic methods and RNA sequencing approaches. The age distribution for DNA methylation analyses skewed towards 0–10 years, with efforts underway to recruit more school-age and adolescent patients. Primary outcomes of interest were tagged for future integration of multiomic data and clinical outcomes, e.g., complicated course (composite of day 28 mortality or day 7 multi-organ dysfunction). DNA QC showed high-quality data.

The team further prepared for analyzing differentially methylated regions (DMRs) in the dataset by normalization of the raw data. Normalization of the variable hyper-methylated peak to the invariable hypo-methylated peak effectively minimized batch effects. Additional sources of variation were removed by adjusting for SNP-affected CpG probes, probes associated with sex chromosomes, and cross-reactive probes. However, there are no healthy controls represented in the dataset, which is a shortcoming. In the final four months of the R21 phase, the team will focus on identifying DMRs that distinguish sepsis from non-sepsis, as well as those that indicate organ dysfunction. They will compare their data to public datasets from healthy pediatric controls. Finally, they will integrate RNAseq and DNA methylation data, and conduct power calculations to identify novel endotypes, while preparing for the larger sample size needed in the R33 phase.

The team learned the value of having high-quality, temporally paired samples and the importance of sensitivity analyses with and without data amputation to avoid false signals. They recognized the need to scale up and validate preliminary findings. Key gaps remain, including studying other epigenomic changes like histone modifications and the need for cell-specific data, since current DNA methylation profiling uses bulk RNAseq. Dr. Atreya will leverage his other R21 project on single-cell approaches to address these gaps. Understanding these upstream epigenomic changes could lead to new therapeutic targets for sepsis, as seen with DNA methylation inhibitors in cancer.

During the Q&A, Dr. Foster asked which blood cell types contributed to the methylation data. Dr. Atreya explained that DNA may be primarily from neutrophils, monocytes, and T cells, with negligible contribution from erythrocytes and platelets. Their bulk RNAseq results indicate overly active neutrophils and neutrophil progenitors with concomitant repression of adaptive immune signatures in septic patients with organ dysfunction. Future single-cell ATACseq studies will clarify cell-specific origins. Dr. Bhattacharyya inquired about detecting non-blood or organ-specific

methylation signatures in bulk samples; Dr. Atreya responded that this is unlikely due to the predominance of whole blood cell signals and overlapping methylation patterns, though future work aims to identify key modifiable upstream factors, despite the source.

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., Emory University School of Medicine
Rishikesan Kamaleswaran, Ph.D., Associate Professor, Duke University School of Medicine

The project introduced by Dr. Esper and Dr. Rishikesan Kamaleswaran, with contributions from specialists in pre-hospital sepsis, machine learning, and VOCs, has three main goals: adopting a clinical sepsis screening algorithm to enroll patients early in their disease course, designing a novel biospecimen collection among sepsis populations in both ambulance and hospital environments, and developing a novel approach to consenting for the sepsis biorepository. Previously, the team was screening sepsis patients in the ICU at one Emory hospital, obtaining blood samples with patient or surrogate consent, or a consent waiver if needed, typically collecting samples at one time point. In this R21 project, they now aim to improve their biorepository by expanding enrollment sites, enhancing screening and sample collection methods, and developing a better consent process.

The team spent six months deploying the screening algorithm across Emory systems, followed by a six-month implementation phase at Grady Memorial Hospital to harmonize retrospective data elements with EPIC EMR mappings, identify sepsis patients, and collect biospecimens. Pre-hospital specimens were collected through a sepsis alert in EMS, including environmental VOCs and blood samples, and then the sepsis screening algorithm in the hospital will identify patients for collecting skin VOCs and blood samples at multiple time points. Fresh blood samples were cryopreserved for RNAseq later. To improve the consent process, they conducted formative interviews with patients and family members and formed a patient advisory panel to understand their views on consent materials. The team will then use that information to develop an improved consent, and then use a survey to look at the impact of the new consent after implementation of the revised consent.

The team made significant progress. First, to develop an early sepsis alert, they tested the impact of moving the currently deployed ICU sepsis alert to the ED, after some key adjustments to the algorithm's variables, such as EtCO2, platelets, creatinine, total bilirubin, and respiration rate per minute. The algorithm was also adjusted to suppress alerts for younger patients under 36 years old, who were less likely to have sepsis. As a result, the algorithm's performance in ED was not negatively impacted, as measured by metrics such as AUROC and PPV. The Algorithm-generated Sepsis Prediction Scores are displayed in the EPIC EMR as a Patient Table, allowing clinical coordinators to identify patients at the highest risk of sepsis.

Notably, the algorithm identified a previously unrecognized group of patients who might have been missed because they were not yet on antibiotics or had not had a blood culture ordered, which not only enhances early sepsis recognition in the ED environment but also improves the timing of sample collection. Generally, the team can collect blood samples within 8 hours of triggering the sepsis alert and send them to the core facility on the main campus for cell isolation and cryopreservation. The preserved PBMC cells have shown up to 98% viability across the 15 samples tested.

One significant challenge the team experienced is that the method they chose to collect pre-hospital VOC samples at EMS, i.e., Tedlar bags, is infeasible due to time constraints during emergency transport. Collaborating with colleagues at Georgia Tech, they developed a rapid canister method for

VOC collection, which is yet to be tested. On the other hand, skin-based VOC collection in the ICU showed promise, revealing distinct metabolite patterns that could differentiate sepsis patients.

Regarding the third objective, the team restructured the consent process based on feedback from patient and family member interviews. They learned that the length, timing, and informational content of the consent process were critical factors for participants. The revised consent process emphasized delayed consent, early communication, the use of simplified patient-centered materials, and continuing evaluation to enhance understanding and trust in biorepository research. They are currently evaluating the impact of the new consent process using a survey.

Lessons learned from the project included the importance of integrating algorithms into clinical workflows and creating an efficient screening, enrollment, and sample collection workflow. The team had challenges in collecting VOC samples, but they learned a lot and were able to develop a new method to collect these samples and came up with a workflow that could potentially work with the EMS team. Finally, improving the consent method will definitely benefit the enrollment. Overall, the study explored and advanced opportunities for earlier and more efficient sepsis screening, innovative sample collection, and more patient-centered consent processes.

Dr. Ware inquired whether the simplified consent form had received IRB approval, noting the presence of IRB-required boilerplate language. Dr. Esper acknowledged this challenge, explaining that the team had to clarify the necessity of this language to the patient advisory panels, and they are communicating with the IRB committee, aiming to achieve the desired balance. Dr. Filbin commented on the importance of a systematic approach to studying the consent process and asked about the timing and performance of the detection algorithm. Dr. Kamaleswaran explained that the algorithm aimed to detect sepsis within six hours of ED admission and before lab results were available, with an AUC of 0.88 based on the Sepsis-3 criteria.

Session I Panel Discussion

Moderators: *Lorraine Ware, M.D., Fran Balamuth, M.D., Ph.D., Leopoldo N. Segal, M.D., M.S.*

The moderators offered the following themes as a starting point for the discussion:

- Challenges in meeting recruitment targets and project milestones
- Pediatric-specific considerations in creating a sepsis biorepository
- Best practices for collecting, preserving, and sharing samples and clinical data

A number of topics were discussed during the panel discussion, as summarized below.

Informed Consent The panel discussion started with people's experience with the different forms of informed consent process, specifically addressing. Dr. Balamuth asked whether any panel members had engaged in discussions with patients and families regarding Exception from Informed Consent Research (EFIC), a method endorsed by the FDA in the U.S., while deferred or delayed consent is less commonly used in the US as compared to other countries. She is wondering if community consultation and public disclosure of EFIC impact their consent decision.

Dr. Esper replied that their team had involved the patient advisory panel in discussions about various consent methods. The panel exhibited mixed opinions but generally supported the concept of simplified consent, provided that the family understood the research, privacy was maintained, and they trusted the approach and procedure of the proposed research. Dr. Yehya Nadir thinks that delayed consent for sample collection has been commonly adopted among investigators. He

emphasized the importance of communicating with the IRB committees about successful prior experience and publishing consent processes to provide references. Dr. Nadir argued that delayed consent helps avoid biased cohorts by including severely ill patients and those less inclined to trust the healthcare system, particularly in the pediatric population.

Dr. Esper noted an additional challenge with the EFIC approach, which is to obtain multiple subsequent samples through informed consent, as patients are still acutely ill, and family members are stressed. Dr. Nathan Shapiro added that their IRB had restricted EFIC to studies with direct benefits to patients, excluding sample collections, so they use a delayed consent. Dr. Balamuth's team had the same experience, so they only use EFIC for interventional studies. For observational studies, they use a waiver of consent for initial samples, followed by prospective consent for subsequent samples. Their team also used a hybrid consent method in a recently completed international trial, while the US site used EFIC, and international sites used delayed consent. Results of this study are expected in the coming year.

Remnant Samples Collecting remnant samples could be a way to avoid complicated consent processes. Dr. Yehya persuaded the IRB committee by arguing the feasibility of collecting clinical information through chart reviews without linking to the patient. They successfully collected remnant plasma samples primarily intended for CBCs and coagulation assays, which have been stored for 24-72 hours. Despite limitations for high-dimensional analysis, he managed to conduct proteomics on these samples and is exploring metabolic studies. Dr. Shapiro noted that their team developed a process to collect fresh remnant samples immediately after clinical assays. He thinks a more unified approach would be great. Dr. Kathleen Stringer cautioned that remnant sample storage duration could introduce analytical variations, particularly in metabolomics, and that they may be more suitable for pilot studies but not confirmation studies.

Dr. Seymour reported findings from his R21 on remnant samples, highlighting reliable protein biomarkers, proteomics, and lipidomics, but significant variability in untargeted metabolomics from samples stored at -4°C. His local IRB committee dictates the length clinical samples are to be stored before collecting remnant samples. He advised collaboration with local IRBs to determine allowable information collection prior to informed consent, including sample storage time.

Sample collection, aliquot, utilization, and storage Dr. Foster raised concerns about aliquoting blood samples for distribution without multiple freeze-thaw cycles. Dr. Segal identified funding as a primary challenge for increased aliquots, hoping the APS consortium could optimize aliquoting practices to foster collaborations. Liquid nitrogen tanks are a more efficient storage method than -80°C freezers due to their low cost and occupying less space. Dr. Ware discussed APS consortium practices, which involve collecting various types of samples from 4,000 patients across >20 sites. APS investigators are grappling with the tension between sample volume, number of aliquots, and length of storage. She advocates for innovative cost-effective storage solutions and mentioned an example of storing plasma samples in long tubes in liquid nitrogen, allowing for piece-wise usage without thawing the entire tube.

Dr. Seymour reflected on the evolution of clinical trial case report forms, noting a shift from extensive forms to single-page formats in some recent trials. He questioned the necessity of collecting extensive types of samples and clinical data, given limited funding and storage constraints. He highlighted the tension between over-collecting and preserving freezer space for new samples, especially when the samples were not utilized fast enough. On the other hand, he noted the benefits of over-collecting in selecting patients of interest retrospectively. Dr. Ware's previous experience mirrored that of Dr. Seymour. She added that a good number of samples that are being collected in the APS consortium have a designated scientific purpose, and the rest are for the biobank and wide

sharing. Dr. Zhao posted a NIGMS NOTICE to encourage the utilization of Data and Biospecimens generated by the APS consortium, i.e., NOT-GM-24-018.

Dr. Kamaleswaran likened biorepositories to living organisms, constantly evolving and connecting rather than static resources. He emphasized thoughtful decisions based on existing data and predicted future utility. Dr. Filbin agreed, advocating for investigators to check existing sample collections before initiating new ones.

Samples Collection in Clinical Trials Dr. Atreya underscored the value of samples collected in randomized control trials (RCTs), which offer insights into patient responses to treatments. Dr. Ware concurred, citing foundational work on ARDS phenotypes derived from NHLBI ARDS Clinical Trial Network biospecimens. She advocated for incorporating sample collection in all clinical trials to leverage patient enrollment and characterization efforts, even without a specific scientific purpose to use these samples.

Dr. Atreya noted a gap in pediatric sepsis clinical trials, with few ongoing studies such as SHIPSS and PRECISE. He called for reconsideration of biospecimen collection in NIGMS-funded trials to explore treatment biology. Dr. Foster highlighted challenges with industry-sponsored trials regarding sample ownership and regulatory requirements. Chat discussions noted the increased regulatory burdens in designing clinical trials, such as the report requirement in clinicaltrials.gov, but admitted that such requirements also serve the purpose of disseminating new trial information.

The panel touched on the usefulness of simulated clinical trials to increase confidence in actual trials. Dr. Seymour proposed an embedded EHR repository to inform biospecimen trajectories. When coupled with target trial emulation and causal inference, it may reveal underlying biology. Dr. Kamaleswaran acknowledged challenges with critical care simulation trials due to irregular observational time series and difficulties in finding a perpetual score-matched individual. Continuous data may ameliorate this problem, but it is mathematically expensive to compute, and the type of available continuous data is biased, creating a distinct Bayesian pitfall in the calculation.

Pre-Disease Samples Dr. Zhao inquired about opportunities to collect pre-disease samples as a control, which is a gap in answering sepsis-related biological questions. Dr. Balamuth highlighted the dilemma of choosing the right timing of sample collection. It is challenging to predict the small percentage of ED patients who later develop sepsis, while starting in the ICU will miss an interesting group of patients who start with infection or sepsis but manage to recover and survive. Dr. Kamaleswaran referenced a publication based on the Glue grant showing differential gene expression within 24 hours of acute injury, stressing the importance of early host response.

Dr. Bhattacharyya discussed efforts to catch pre-disease indicators of sepsis, citing a study on Gambian neonates that identified transcriptional signatures on the day of birth predictive of sepsis. He asked about the incidence of sepsis in Dr. Prescott's cohort. Dr. Prescott shared that most of their cases are early-onset sepsis caused by issues during delivery, and she believes more could be learned from analyzing late-onset sepsis cases (~15% of the cohort), i.e., culture-negative when enrolled but become septic later. They collect several weeks of normal samples before they get the late-onset sepsis. There is also a group of patients who were initially ill but recovered after receiving antibiotic treatment. The caveat is that most preemie babies were indirectly exposed to antibiotics through their mothers, and that the definition of sepsis in the NICU differs from other age groups, i.e., bacteremia but not necessarily with organ dysfunction, whereas in older children and adults, organ dysfunction is key to a sepsis definition.

Dr. Seymour emphasized the ideal opportunity to collect healthy samples in neonatal populations, using the example of heel stick newborn screening, such as the Acylcarnitine screening. For adult patients, perhaps at-home sampling is an opportunity to get pre-disease samples. Dried blood sampling may be useful in this case. There are also some early technologies supported by industry and BARDA to identify SIRS patients before they become septic. Dr. Filbin supported over-sampling in emergency settings to capture initial samples, given the difficulty in predicting sepsis in the ED.

Program integration Dr. Balamuth inquired about the potential integration between the sepsis biospecimens program and the APS consortium, highlighting the possibility of leveraging synergies between the two initiatives. Dr. Ware, who is involved in both programs, shared that her experience with the sepsis biospecimens project had significantly influenced the development of standard operating procedures for the APS consortium. However, she pointed out a key difference: the APS consortium focuses exclusively on adults, lacking a pediatric perspective, which represents a significant gap. Dr. Ware expressed her appreciation for the extensive pediatric work being conducted in the sepsis biospecimens program, noting the discussed advantages of this cohort in identifying common mechanisms of sepsis.

Session II: Novel Samples, Biomarkers, and Analytic Techniques

Session Co-Chairs:

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

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

Dr. Filbin introduced the Session II co-chairs: Matt Foster from Duke University School of Medicine and Dr. Cristina M. Furdui from Wake Forest University School of Medicine. Drs. Furdui and Foster introduced the speakers and moderated the Q&A following each presentation.

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

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

Dr. Foster's project, supported by MPI Dr. Tim McMahon and funded by NIGMS, began in 2020 during the COVID-19 pandemic. The primary goal was to develop a method for sampling small volumes of blood (10-30 µl) using MITRA devices. These devices were initially designed for remote sampling via fingersticks, but the team adapted them to dip into EDTA blood tubes, dry the samples for two hours, and store them at -80°C. The use of dried blood aimed to capture cellular components that are often missed in serum-based proteomics and metabolomics, reduce pre-analytical variability, and study post-translational modifications extensively.

During the R21 phase, the team focused on method development, achieving milestones such as proteomic characterization of proteins, glycopeptides, phosphopeptides, and metabolites using two MITRA devices. They conducted ex vivo stimulation experiments for assay validation and tested analyte stability under different storage conditions. Scalability for processing hundreds of samples simultaneously was also assessed. In the R33 phase, the team plans to scale up assays to about 700 patient time points, develop best practices for data reporting, integrate results into clinical data, and disseminate the data for future hypothesis generation.

They employed a detailed sample processing workflow for non-targeted proteomics assays, involving a ~4 h-long in-solution digestion of Mitra tips in matrix tubes, followed by the enrichment of glycol-peptides and phospho-peptides. This entire process can be completed in a 96-well format within two

days. The team utilizes commercial software (Spectronaut) for analyzing the proteome and phosphoproteome, and academic software (GIyco-Decipher and FragPipe) to identify and quantify glycopeptides. They also tested scaling up from a 96-well to a 384-well format, using advanced instruments like Microflow LC for proteome analysis and Evosep One for PTMs. They performed data cleaning to remove duplication and accurately report phosphorylation sites. Data was uploaded to Proteome Xchange, and several manuscripts are in preparation. They also conducted metabolomics and sent datasets to NIH collaborators for analysis.

The team analyzed samples from two studies, first the SICK (SepsIs Characterization in Kilimanjaro) study led by PI Matt Rubach and funded by NIAID (R01AI155733). This study enrolled 500 patients under the sepsis-2 definition and collected samples at days 0, 1, 3, and 28, while MITRA dried blood samples were available from 38 patients, and a subset was selected to fill a 96-well plate for proof-of-concept purposes in the R33 phase. The team used a pooled sample control (SPQC), which should sit at 0 on a PCA because it is the average of all the samples. They also had a healthy control pool from blood type-matched individuals staggered across the 96-well plate. Another quality control measure was to compare Mitra CRP levels and clinical assays of CRP, which showed a strong correlation.

They were able to quantify 2,000 proteins, 3,750 N-glycopeptides, and 5,550 phosphosites, exceeding the preset milestones. The data, along with some clinical metadata, have been uploaded to Proteome Xchange (PXD060377), and the results are available in a preprint. Instead of generating 3 separate PCAs of protein, N-glycopeptides, and phosphosites, the team put all the data into one factor analysis using the multiomics factor (MOFA) 2 package. The multiomic latent factors plot shows which proteomes contribute to each factor, allowing investigators to identify the specific features associated with each omic's weighting factor. For example, CRP weighed the highest in the proteome group; α1-antichymotrypsin weighed the highest for the glycopeptides, and for phosphor-peptides, osteopontin. These proteins have been well described in sepsis, so the data make sense as proof of concept. Comparing samples from day 0 to day 28, they observed lower levels of acute phase proteins and neutrophil-derived proteins on day 28, and regression analysis identified correlations between certain proteins (Kallistatin and Clusterin as negative correlators and Cytochrome C and osteopontin as positive correlators) and Universal Vital Assessment (UVA) scores (a clinical variable).

The second study the team analyzed is the STAR study (R01HL161070, PI: Tim McMahon), which recruited patients under the Sepsis-3 definition and performed ex vivo manipulation of RBC ATP using different approaches (pyruvate kinase activators, PIPA, hypoxic red cell storage). The latent factors plot showed a nice separation of the sepsis vs non-sepsis samples. They found similar time trajectories and weight factors for the proteome, glycol, and phosphopeptides as in the SICK study. Elevated levels of α1-antichymotrypsin in septic samples compared to controls were confirmed. Currently in the R33 phase, the team is analyzing additional STAR batches, analyzing non-stimulated and stimulated samples, and working with a post-COVID biobank of ~384 samples with long-term follow-up for up to 12 months, as well as conducting prospective validation for the above results. They are generating hypotheses and have observed promising signals for detecting sepsis and tracking recovery. Efforts are ongoing to integrate clinical data and optimize data analysis pipelines to make it more accessible, including the use of dashboards for data visualization.

Dr. Foster summarized the lessons and challenges. Efficient sample processing would benefit from minimal sample handling, using study-specific pools, and continuous improvement in protocols. For example, they streamlined sample cleanup by using disposable trap columns (Evotips) that avoid extra steps of drying and reconstituting. Challenges included metabolomics analysis complexities

and the need to convince other researchers of the benefits of using dried whole blood for omics studies. In their experience, MITRA tip sampling is highly compatible with biobanking protocols, and the team is seeking collaboration opportunities.

In the Q&A session, Dr. Yehya asked about the mass spec method's ability to detect intracellular signals and the impact of cell death during sample processing. Dr. Foster explained that their sampling is upstream of and unaffected by plasma processing and that whole blood MITRA tip samples showed less pre-analytic variation compared to plasma. However, they cannot differentiate the source of proteins. Dr. Segal inquired about capturing the cell composition of PBMCs, which could be used to adjust omic analysis for leukopenic or leukocytotic patients. Dr. Foster said that data from Tanzania typically have sparse white blood cell counts and agreed that expressing data relative to leukocyte counts would be useful.

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

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

In this project, Dr. Bhattacharyya, an infectious disease physician at MGH and an assistant professor at Harvard Medical School and the Broad Institute, collaborates with Drs. Michael Filbin and Nathan Shapiro. It focused on the cryopreservation of whole blood for single-cell sequencing. The primary motivation behind this project is the increasing feasibility and value of multiomic assays at single-cell resolution. The project aims to make the labor-intensive sample processing for scRNAseq more accessible and properly integrated into clinical workflows.

The single-cell approach offers a transformative approach for understanding cellular and, therefore, mechanistic context on top of bulk sequencing-based endotypes and finding targetable mechanisms. It also avoids misleading results by averaging measurements of a very diverse cell population, like getting Pegasus by averaging a population of farm animals and poultry. Single-cell measurement has revolutionized how people think of heterogeneity in a lot of complex systems, from oncology to neuroscience to developmental biology. Importantly, it has yielded a lot of information about the immune system at a resolution that was not available a decade ago. It is particularly attractive for sepsis since the disease is caused by distinct or opposing immunopathology in different patients.

The team has shown previously that resolving cellular heterogeneity of the immune response to sepsis can provide meaningful insights into patient-level heterogeneity. In a pilot study with a clean cohort of urosepsis patients and UTI controls, they performed PBMC scRNAseq and cell clustering on UMAP. Subclustering each of the immune cell lineages revealed even more information, which revealed four distinct transcriptional substates of monocytes, including a unique type (MS1) almost exclusively found in sepsis patients, sometimes accounting for >50% of their PBMCs. Subsequently, the team extended their study to include samples from hundreds of sepsis patients with diverse causes and found the MS1 cell type in all of them, including COVID-19 sepsis patients, where it was the best predictor of disease severity. They are now diving deeper into the biology of MS1 cells.

The Cryo-PRO method developed in this project offers a simple and efficient protocol for sample processing. This has advantages over the traditional Ficoll method to isolate PBMCs, which requires ~2h at bedside using <2 ml of whole blood, as well as specialized expertise and equipment, making it difficult to integrate into clinical settings. The Cryo-PRO method involves adding a cryoprotection step using 10% DMSO, immediately freezing the samples at -80°C, and centralized and batched sample processing. It reduces bedside processing time to <10 min in simple steps and minimizes variability. The team optimized methods for post-thawing cell depletion, which is where the innovation of this project lies. They found that antibody-based magnetic depletion of red blood cells

is the most effective, followed by the normal process of flow cytometry for PBMC isolation, as summarized in a preprint. Batched sample thawing and sequencing (e.g., 8 samples) saved even more processing time and delayed processing for at least a month in -80°C and longer in liquid nitrogen also offers convenience to the researchers.

This method was tested in 32 samples of 24 patients recruited from two sites. Compared to the Ficoll method, Cryo-PRO produced similar cell viability and high-quality scRNAseq and CITE-seq data. The two methods yield similar patterns of percentage cells expressing key markers in specific cell populations, while there were some differences in freeze-thawing sensitive platelets and stress response genes, such as JUN and CXCL8, both higher in the Ficoll group.

Overall, Cryo-PRO facilitates the integration of scRNAseq in clinical studies. It reduces resources required at the bedside and aligns well with the delayed consent process, so the samples are processed only when consent is obtained. It also increases the feasibility of integrating single-cell studies into randomized controlled trials, while post hoc analysis of differential treatment effects at the single-cell level is quite exciting. In the R33 phase, the team is excited to integrate this study as an ancillary study of the APS consortium to expand the capacity to do single-cell sequencing in the consortium. It plans to process 500 samples, by far the largest single-cell study in sepsis. They will work on streamlining the centralized processing step through parallel flow cytometry of multiple samples and functional studies such as ELIspot and response to stimuli.

During Q&A, Dr. Segal highlighted the importance of publishing quality control-related papers to inform the field and added that scRNAseq of different sample types behaves distinctly based on his experience with lower airway scRNAseq. He pointed out the differences in transcripts observed between the two methods and wondered if these were due to changes in cell subtypes. Dr. Bhattacharyya explained that the differences were mainly due to the minor stress response induced by the Ficoll method. Dr. Ware is excited about the Cryo-PRO method as an ancillary study of the APS site. She asked if there were any plans to preload the sample tubes with DMSO to make the process even easier. Dr. Bhattacharyya thinks it is a good idea for fixed volume sample collection and mentioned their ongoing tests for how varying percentages of DMSO affect cell behaviors.

Redox Trapping for Biospecimen Preservation and Innovation in Sepsis Care

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

Dr. Furdui and her colleague, Dr. Files, presented their R33 project to develop a new formulation that preserves the redox state of blood. This is because the dynamics of redox metabolism are closely linked to responses to infections, and progression to sepsis and septic shock. The team decided to focus on blood samples due to their easy access, richness in biological information, and popularity in diagnostic use. Yet, blood is susceptible to oxidative damage, and common methods for blood collection do not try to preserve the redox state in PBMCs or in the red blood cells (except for the use of NaF/KOx additives in blood collected for glucose test). A redox trapping formula would thus prevent oxidative damage and halt metabolism. It is promising in revolutionizing biomarker discovery in sepsis and beyond, reducing pre-analytical variability, and advancing clinical research.

The team had three principles when designing the redox trapping formula: preventing the artificial formation of reactive oxygen species through inhibiting Fenton reactions, stopping the propagation of single electron/radical reactions (e.g., lipid peroxidation), and protecting macromolecules from damage caused by reactive oxygen species (e.g., thiol blockers). The ingredients should also not interfere with the current gold standard clinical lab assays and have a relatively low cost.

They chose specific biomarkers to measure the efficacy of their redox formulation, including Peroxidoxins (Prx) and Glutathione ratio (GSH/GSSG), with higher monomeric Prx and GSH/GSSG signifying better redox quenching capacity. Prx is measured by SDS-page while GSH/GSSG is quantified by Mass Spectrometry. Their preliminary data demonstrated that their formulation RMX could effectively quench redox reactions in varying blood volumes, as indicated by similar levels of Prx monomers with 1 ml vs. 1.25 ml of blood. In addition, the RMX mixture improved the quality of specimens during freeze-thaw cycles and long-term storage, as demonstrated by a significant reduction in VEGF degradation, a known biomarker for protein stability during freeze-thaw cycles, particularly for samples stored at -80°C for up to 12 months. The cost is fairly low, <$0.72 - $2.5 (add protease inhibitor) / 3 ml tube.

In the R21 phase, the team initiated the REDOX SEPSIS I study, enrolling sepsis patients and testing the redox formulation for various clinical and research laboratory measurements. Data from this study indicated that the formulation improved the stability of lactic acid at room temperature and did not interfere with clinical lab assays such as CMP and CBC panels, although it affected the measurement of calcium and some ions due to the presence of metal ion chelators. RMX also significantly reduced the GSH/GSSG ratio (<1 without RMX vs ~2,000 with RMX). A similar improvement in redox state was shown using the Prx assay, i.e., more Prx monomers with added RMX. This result indicates that RMX can stop metabolism (better than NaF/KOx) and reduce sample oxidation.

Dr. Files explained that in the R33 phase, they will enroll 150 patients under the Sepsis-3 definition using formal informed consent in 5 sites. The cohort has good geographic representation and includes 3 strata, i.e., sepsis patients, infected controls, and septic shock patients, aiming to capture the dynamic transitions from infection to sepsis and septic. Each patient was assigned to an enrollment stratum early (<72h in hospital) under broad inclusion criteria and a post-hoc stratum based on adjudication at the end study (D7 in hospital). They had enrolled 18 patients so far, with an estimated completion date by the end of 2025. They collect plasma, PBMCs, and RBC in aliquots using the Ficoll method and will perform multiomics and examine the redox state with or without RMX.

Overall, RMX demonstrated promising value both to enable clinical research on redox metabolism and to help with clinical care by improving lab tests such as lactate.

During the Q&A session, Dr. Foster inquired if the redox formulation, RMX, could help stabilize dry blood samples for metabolomics and other applications. Dr. Furdui expressed interest in testing this and in Cryo-PRO samples, believing that RMX ingredients should not interfere with proteomics or phosphoproteomics. Comments in the chat questioned whether the formulation could differentiate intracellular versus extracellular origins of molecules, given the more reduced intracellular space. Dr. Furdui responded that comparing plasma versus whole blood lysate could reveal differences in the redox state of key biomarkers, potentially providing valuable insights in this regard.

Dimethylmethylene Blue as a Rapid Assay for Circulating Glycosaminoglycans in Sepsis

Eric P. Schmidt, M.D., Massachusetts General Hospital and Harvard Medical School

Dr. Schmidt introduced himself and Dr. Nathan Shapiro at Beth Israel Deaconess Medical Center, the MPIs of the project. The primary goal of their research is to develop a point-of-care assay to measure endothelial glycocalyx degradation at the bedside, aiding clinical treatment decisions for sepsis.

Dr. Schmidt first elaborated the premise of the study. Endothelial glycocalyx is a significant intravascular extracellular matrix extending from the endothelium into the lumen, composed of cell-

surface proteoglycans and glycosylated glycans, which mainly include heparan sulfate (HS) and chondroitin sulfate (CS). HS, which contains repeating disaccharide units that can be sulfated at specific locations of the sugar ring (6S, 2S, or NS) and thus possesses patterns of negative charges that bind to positively charged proteins, plays crucial roles in endothelial barrier function, mechanotransduction, leukocyte adhesion regulation, and preventing intravascular coagulation. Dr. Schmidt's lab has shown that sepsis degrades the endothelial glycocalyx via heparanase, releasing HS fragments and attached proteins into the bloodstream. This degradation leads to endothelial dysfunction and subsequent lung and kidney injuries in both animal models and human studies. The team was able to detect fragmented HS in the plasma of septic patients for up to seven days. They developed methods to quantitatively measure HS and conducted a secondary analysis of 574 patients from the CLOVERS clinical trial, which assessed the effectiveness of different fluid resuscitation strategies in sepsis patients. Although the trial was stopped early due to futility, the early biospecimen collection allowed the team to explore the prognostic value of glycans in sepsis.

Using state-of-the-art mass spectrometry, the team measured glycocalyx degradation in 580 CLOVERS patients at three time points within the first 72 hours of enrollment. Their findings revealed that HS levels in the blood were a powerful prognostic biomarker for sepsis mortality, with multivariate analyses confirming HS as a top predictor of 90-day mortality. This suggests that sepsis may be an endothelial disease rather than purely an inflammatory one, as HS levels were more predictive of mortality than IL-6. They also measured CS levels, initially not expecting much due to its location at the bottom of the glycocalyx chain and its constant presence in plasma from other sources (e.g., bikunin/IαI). Surprisingly, they found that CS levels could predict patient response to fluid resuscitation, with lower CS levels correlating with better outcomes in liberal fluid strategies and higher CS levels correlating with better outcomes in restrictive fluid strategies.

The team is currently working on translating these findings into clinical care. While mass spectrometry is the gold standard for measuring HS/CS, it is expensive and laborious. They are developing a simpler, faster, and cheaper test using the Dimethylmethylene blue (DMMB) assay, originally designed to detect sulfated glycosaminoglycan fragments in synovial fluid. The DMMB assay is a colorimetric test that binds to sulfated glycosaminoglycans and darkens proportionally to the level of sulfation. The team optimized the assay for airspace fluid and urine samples previously, demonstrating its ability to detect both HS and CS. To reduce the background of the DMMB assay, they added a dilution step to normalize baseline color levels and titrated down the pH of the reagent to reduce sensitivity to the negatively charged cell-free DNA while maintaining sensitivity to glycans.

In their R21 project, the team confirmed the linearity of the DMMB assay using water, pooled healthy plasma, and pooled septic plasma spiked with known amounts of CS. They found that the assay could be used for blood samples, with the exception that spiked septic plasma saturated at high CS levels, while the addition of cell-free hemoglobin and bilirubin did not confound DMMB results significantly. Side-by-side comparison of DMMB and mass spectrometry data on 283 banked sepsis patient samples showed a significant association for both HS and CS, while HS had more biological variability. To address this, they treated samples with proteases to remove hypothetical proteins bound to the HS/CS, which improved the association. Previous research showed that HS quickly excreted into the urine, so the team also tested whether urine DMMB could quantify plasma glycosaminoglycans. Initial results showed an association between normalized urine DMMB to creatinine and plasma HS levels, but not for CS.

Overall, Dr. Schmidt's team showed that plasma glycosaminoglycans can be both a prognostic (HS) and predictive (CS) biomarker for sepsis, which could be a powerful tool for precision medicine in sepsis. They are developing an inexpensive, rapid point-of-care assay for detecting plasma CS and urine HS, which could enable precision medicine approaches in sepsis.

During Q&A, Dr. Ware asked how the DMMB assay differentiates HS from CS and why they are not combined in analysis. Dr. Schmidt explained that the assay captures any sulfate strings and that combining HS and CS would reduce the distinct prognostic and predictive values. Dr. Nadir inquired about correlating the DMMB assay with protein markers like syndecan or angiopoietins. Dr. Schmidt noted that HS levels, but not CS, match syndecan-1, perhaps due to the high background level of CS in the plasma. Both are imperfectly associated with endothelial injury biomarkers.

Dr. Zhao asked whether the CS data predicting fluid resuscitation responses were averaged, and how many biological variations there were. Dr. Schmidt responded that the CS prediction was normalized by multivariate factors such as age, severity, and comorbidity, which showed a strong association with fluid strategy responses, sparking interest in the underlying mechanisms. Dr. Zhao suggested collaborating with a biochemist to develop a more differentiating point-of-care assay. Dr. Schmidt speculated that similar strategies to those of the Thin Layer Chromatography to separate components in a mixture could potentially work. A question in the chat wondered whether circulating CS was from leaking vessels. Dr. Schmidt mentioned a small study that found no association between lung edema and plasma CS in ARDS patients, highlighting the need for further research.

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

Basilia Zingarelli, M.D., Ph.D., Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine

Dr. Zingarelli introduced the R21 project and her MPI, Dr. Jennifer Kaplan. The project focuses on extracellular vesicles (EVs), also known as exosomes, in the context of sepsis. EVs are lipid bilayer membrane nanovesicles that range from 40 to 150 nanometers in size and are released into the extracellular environment by almost all cell types. These vesicles carry various components, including membrane markers and unique cargos derived from the cytoplasm, such as DNA/RNAs, proteins, lipids, and metabolites. Notably, EVs serve as the main reservoir for extracellular RNAs, particularly microRNAs, and play crucial roles in intercellular communication. By influencing recipient cells through direct receptor activation or cargo release, EVs can induce epigenetic, pro-inflammatory, or anti-inflammatory changes. This mechanism is observed in both physiological processes, like maintaining cell homeostasis, and in disease processes, including sepsis.

The team's ultimate goal is to explore the potential of EVs in clinical diagnosis and their role in understanding the molecular mechanisms of organ injury in sepsis. Despite their promise, challenges exist in using EVs in sepsis research, such as the lack of standardized isolation and purification methods, difficulty in accurately identifying EV-specific biomarkers, limited understanding of their complex molecular cargo, and limited sample volumes in pediatric research. To address these gaps, Dr. Zingarelli's team leveraged their existing resources. One is a pediatric sepsis biobank, which contains over 2,300 patients with de-identified demographic and clinical data and more than 26,000 aliquots of various samples. Over half of these patients were stratified through the severity risk model PERSEVERE-II, and a portion of them also had biological biomarkers measured. The group also invested in a scientific data management system called the LabKey, which not only provides a central digital repository for all data but also serves as an integration platform for combining clinical data and biological data, affording real-time data analysis.

Utilizing these resources, the team completed two specific aims. First, they optimized methods for sEV collection and isolation from plasma and serum samples of septic subjects. They tested the physical characterization of the resulting sEVs by nanoparticle tracking analysis and assessed their

suitability for RNA extraction and RNA sequencing. The team found that the PS-affinity extraction method produced purer and more homogeneous sEVs than ultracentrifugation, but produced sEVs ranging from 40 – 150 nm in size, as expected. Negative staining at transmission electron microscopy confirmed the spheroid and membrane-bound shapes of the sEVs, with PS-affinity extracted sEVs showing more homogeneous shapes and consistent presence of sEV content. The PS-affinity method also yielded higher particle concentration, indicating a higher yield. Interestingly, serum EVs from sepsis patients were significantly smaller compared to controls, possibly due to differences in the cell of origin.

The team also showed that EVs have immunomodulatory characteristics, as seen in their experiments with THP1-Dual cells that are transfected with reporters of the NF-κB and the IRF pathways. sEVs did not induce cell death alone or in combination with LPS and could increase cell metabolic activity in a concentration-dependent manner. While sEVs alone did not activate NF-κB or IRF responses in THP1 cells, they altered NF-κB responses in cells exposed to LPS but had little to no effect on IRF responses induced by LPS. sEVs from control subjects inhibited NF-κB activation, while sEVs from septic patients only showed this effect at higher concentrations when isolated from the serum by the PS-affinity method. Ultracentrifugation-isolated serum sEVs showed similar trends but with higher variability. Plasma EVs do not show any immunomodulatory effect. There is also a possible link between the EVs and clinical characteristics. In a pilot test, patients with cardiovascular and respiratory failure had higher levels of serum EV isolated by the PS-affinity method, while the in vitro immunomodulatory functions of serum EVs correlate with lung injury but not cardiovascular injury, maybe due to a different population of EVs produced by these patients.

For the second aim, the team demonstrated the suitability of EVs for high-throughput analyses of RNA cargo. They found that both PS-affinity and ultracentrifugation methods yielded similar RNA integrity and quantity, although the latter produced more heterogeneous results. All samples passed RNA quality control, allowing the team to proceed with small RNA sequencing and bioinformatics analysis. Preliminary findings revealed changes in RNA biotypes during the clinical course of sepsis. For example, the lung-specific Serine/Threonine Kinase 17b only presents in sepsis patients with respiratory failure, while absent in those with only cardiovascular failure. Other hits that correspond to clinical characteristics include proinflammatory mediators, mitochondrial RNA encoding for humanin, and endothelial injury markers.

In the R33 phase, the team plans to further confirm whether sEVs can inform different types of organ injury. They aim to conduct a retrospective full endotyping of sEVs in patients with specific types of organ failure and validate these findings in a prospective study to determine if patients can be classified based on sEV endotypes. The team also intends to explore the characteristics of EVs using their newly acquired analyzer capable of multi-channel fluorescent nanoparticle tracking analysis, allowing for multiparameter characterization of single-EV particles.

Overall, the team showed that the PS affinity method is a simple process that allows for the capture of EVs with high purity using a small volume of samples (250 µl), and they are suitable for functional studies. It is superior compared to the ultracentrifugation method.

During the Q&A session, Dr. Zingarelli addressed several questions. One is about tracking the cell or organ source of EVs. Dr. Zingarelli explained that they could use antibodies for specific markers of the cell of origin to enrich EVs. Another inquired about the identity of the active molecules within the EVs and whether the distribution differs between the two EV isolation methods. Dr. Zingarelli believes that biologically active molecules in EVs may depend on their cell of origin. She thinks that ultracentrifugation does not yield an adequate representation of the different cell heterogeneity of EVs, suggesting that enrichment of EVs may be the answer. Another audience member asked if the

team plans to analyze EVs from serum versus plasma by proteomics to determine the differences. Dr. Zingarelli confirmed that this is in their plan. Finally, a question was raised about the possibility of missing PS-negative EVs with PS-affinity isolation. Dr. Zingarelli acknowledged that PS-negative EVs are a small subpopulation and that they can measure the zeta potential of these EVs, which could inform whether EVs isolated by PS-affinity or ultracentrifugation had different charges.

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

Mihir R. Atreya, M.D., M.P.H., Cincinnati Children's Hospital Medical Center and University of Cincinnati
Andrew J. Lautz, M.D., Cincinnati Children's Hospital Medical Center and University of Cincinnati

Dr. Atreya introduced himself and his MPIs, Drs. Lautz and Zingarelli. The goal of the project is to establish a biobank of patient-specific induced pluripotent stem cells (iPSCs) for mechanistic research into pediatric sepsis. This initiative aims to address current gaps in sepsis research, such as the biological differences across species, the homogeneity of disease models, and the low transduction rates of existing pre-clinical models. Human iPSCs, which can be reprogrammed from somatic cells using Sendai viral reprogramming and Yamanaka factors (Oct4, Sox2, Klf4, C-myc), offer a renewable source of biospecimens. These cells can be differentiated into specific cell types and used in organoid models. The team focuses on disease modeling and drug screening, though iPSCs also have potential for genome editing and cell-based therapies.

The team has made significant progress in the R21 phase of their project. They utilized data from the PERSEVERE study, which identified a set of protein biomarkers for risk stratification of sepsis patients based on the number of organs affected. Human iPSCs were treated with risk-stratified septic samples. Preliminary data showed that treatment of healthy control human iPSC-derived monocytes with high-risk serum enriched their expression of inflammatory genes. Similarly, the treatment of human iPSC-derived endothelial cells in an organoid model by high-risk serum upregulated their glycan signaling, angiotensin signaling, and stress responses. Human iPSC-derived cardiomyocyte spheroids, formed through guided differentiation targeting the Wnt signaling pathway, showed mature sarcomere structures. Treatment with high-risk serum decreased the sharp shortening and beat frequency of these spheroids, as well as altering their transcriptomics. These findings indicate that healthy donor-derived iPSCs exhibit robust responses across cell types when treated with risk-stratified sera from septic patients, providing a valuable disease model.

The project is divided into 4 sub-aims: establishing an enrichment strategy to isolate primary cells, phenotypic and functional validation of human iPSCs in the R21 phase, multiomic comparison of primary and iPSC-derived cells, and establishing a multicenter patient-specific iPSC biobank in the R33 phase. In their single-center prospective observational study, whole blood samples were collected in Acid Citrate Dextrose (ACD) and EDTA tubes at day 1 (within 24h of enrollment) and day 3. All samples were processed within 6 hours of collection.

The team validated a two-step cell enrichment protocol using magnetic separation, with red blood cell antibodies used for RBC depletion, which preserved neutrophils better than the Ficoll method. Sample aliquots were then separated into a CD45+ immune fraction and a CD45-CD34+ circulating endothelial cell fraction. This process generated enriched cells of high quality, confirmed by cell viability and flow cytometry. They established two IRB protocols, one for sample collection and one for iPSC generation. They successfully generated iPSCs from various genes and age groups, with a much larger CD45+ than the CD45-CD34+ fraction. Human iPSCs are available from 8 of the 14 recruited patients, and recruitment is ongoing. They use a pluripotent cell facility and a standardized protocol to develop, validate, and perform quality control for these cell lines.

Key lessons learned include observation that storage in ACD tubes resulted in greater cell viability than EDTA tubes for downstream iPSC generation, with viability also affected by storage temperature. Patient-specific iPSC lines can be generated from both post-RBC depletion fractions and CD45+ fractions. Additionally, patient-specific iPSCs were successfully generated from infants using 3-5 ml of whole blood. However, generating iPSCs from patients with leukopenia at the time of sampling (e.g., bone marrow transplantation, cancer) proved challenging.

Dr. Lautz continued the presentation on aim 2 of the R21 phase, which looked at the genomic and epigenetic characteristics of iPSCs from the control and septic patients. They compared septic patient-specific iPSCs to generic iPSCs banked in their stem cell facility. Dr. Lautz, particularly interested in iPSC-derived cardiomyocytes, treated iPSCs with serum from sepsis-associated cardiovascular dysfunction (SAMD) or low-risk patients. Results showed that SAMD serum exposure reduced contractility in iPSCs from SAMD patients but not in generic iPSC lines, suggesting that genetic components may play a role in the development of cardiovascular dysfunction in septic patients. In the future, the team plans to expand the biobank of patient-specific iPSCs to capture greater genetic or epigenetic heterogeneity, explore the use of this information for high-throughput drug screening for sepsis subtypes, compare iPSCs with primary cells, and establish organ-specific organoid models of dysfunction in sepsis.

Dr. Furdui opened the Q&A session by asking if cryopreservation of iPSCs would alter their viability and functionality. Dr. Atreya responded that they had been focusing on testing freshly isolated samples, and testing cryopreservation of these cells would help with the subsequent utilization of these cell lines. One potential challenge in this process is to further enhance the consent rate, as only a portion of patients agreed to have their iPSCs banked for future research.

Session II Panel Discussion

Moderators: *Michael Filbin, M.D., M.S., Matt Foster, Ph.D., Cristina M. Furdui, Ph.D.*

The moderators offered the following themes as a starting point for the discussion:

- Advantages and pitfalls of novel sampling methods
- Role of novel assays in a sepsis biorepository and/or clinical care
- Key features of the ideal sepsis biorepository: lessons learned from our research and experiences

A number of topics were discussed during the panel discussion, as summarized below.

Managing sample collection, storage, and processing The panel discussion opened with Dr. Filbin encouraging a focus on the common threads across various approaches and how they might be integrated into biobanking and clinical workflows. Building on this, Dr. Furdui highlighted the need to tailor sample storage to downstream analyses, noting that while not all sample components need long-term preservation, some high-value samples like iPSCs and PBMCs retain and should be stored. She suggested a shift toward real-time assays with data storage as a potential strategy. Dr. Foster supported long-term storage of whole blood samples for omics, as this does not compromise the data quality in his experience. Dr. Ware outlined the APS strategy to standardize sample collection protocols and plan analysis before starting to collect. She noted that APS will conduct a robust suite of assays, including scRNAseq on NBBAL and PBMCs, metagenomics, microbiome profiling, and standard protein biomarker studies, all aiming at advancing molecular phenotyping and understanding disease mechanisms. Together, the discussion underscored a shared commitment to strategically aligning sample collection with evolving research and clinical needs.

Reflecting on the new methods presented, Dr. Atreya noted the importance of both mature and early-stage methods for pipeline continuity. Dr. Segal emphasized that research questions should guide methodology development, noting that methods must adapt based on scientific questions, much like a biorepository should adapt like a living organism. He stressed the importance of sharing successful and unsuccessful approaches in this evolving process.

Dr. Furdui raised the topic about the value of different versions of similar types of samples, like plasma and whole blood, in sepsis research. Dr. Foster highlighted the advantage of accumulating large amounts of data from the same samples, which led to reliable reference databases, akin to the work by UK Biobank. Any sample type has pros and cons. For example, whole blood samples catch cellular components of the blood, but freezing and thawing of whole blood will generate potential artifacts from RBC breakage, particularly when measuring metabolites and glutathione. Dr. Bhattacharya added that multiple data sets are crucial, noting that plasma proteins are significantly affected by kidney function, which must be considered in endotyping. Currently, there is a lot more protein marker information than cellular information.

Buy-in for novel methods supported by the sepsis biospecimens program Dr. Foster and Dr. Zhao raised questions about the applicability of novel methods on large patient cohorts, such as the APS consortium, and how to increase buy-in for these methods. Dr. Furdui suggested that awareness is key, noting that their group started with standard methods but is interested in concepts like dry blood sampling and Cryo-PRO to increase efficiency in multi-site phases. Dr. Bhattacharyya added that convincing people to adopt new methods is challenging, as established methods are deeply ingrained in clinical workflows. However, since their Cryo-PRO preprint became online, there are 3 more preprints published on cryopreservation concepts for scRNAseq, which have given them confidence that they are moving towards the right direction. They plan another validation round comparing Cryo-PRO to the CPT method of PMBC isolation before applying it to all APS consortium patients in the R33 phase.

Dr. McMahon wondered if **Cryo-PRO** could be useful for specific patient populations, like those who have thrombolytic diseases. Dr. Bhattacharyya replied that they had mainly tested it on control and sepsis patients, but have colleagues in the cancer field eager to test it in cancer-related prothrombotic states. He noted that Cryo-PRO is not compatible with neutrophils, which are lost during freezing and thawing, though their transcriptomic information is retained in the plasma or serum samples. Dr. Furdui commented that when people isolate single cells from tissues for RNAseq, they also use a step of DMSO cryopreservation, similar to the principle of Cryo-PRO, so this method is potentially generalizable.

Dr. Furdui also discussed whether their **Redox mix** could be applied to other fluids, such as lower airway samples, and its sensitivity in ongoing trials with varying redox status (e.g., patients with various levels of inspired O2). She explained that while they had not tested it on sample types other than serum, they had looked at blood from cancer patients and found that the redox levels in these patient samples can be largely resolved by adding RMX. She is confident that the Redox mix should work with BALF samples and possibly tissue samples when permeabilizing reagents are added.

Dr. Schmidt highlighted that **glycans** are remarkably stable, even under multiple freeze-thaw cycles, with minimal degradation over a long period of time, so people can measure glycans from dinosaur fossils. He mentioned their interest in using glycans in old biobanks due to their heat and temperature resistance. Dr. Foster asked if urine samples were controlled for dilution, to which Dr. Schmidt replied that samples were normalized to creatinine and urine protein levels. He suggested a dip test for urine density to improve assay accuracy.

Dr. Ware inquired about people's experience in the use of **HME filters** in pediatric populations for non-invasive ways to collect lower airway samples. To her knowledge, HME filters are not widely used in this patient population due to the concerns of adding dead space to a relatively smaller volume of ventilation. Dr. Atreya noted that miniBAL and nasal swab samples were more commonly used. Dr. Yehya added that their group found significant variations with HME filters based on humidity and patient diagnosis, leading them to abandon this approach. Dr. Prescott mentioned that in neonates, moisture filters are closer to the ventilator, making them easier to collect, and expressed interest in collaboration for testing these filters. Dr. Ware added that most moisture filters do not release fluids easily, but the AirLife HME filter, using a hygroscopic sponge, seems to work consistently, and a neonatal version is used in their hospital during neonatal transfers. However, HME filters cannot be used with humidified circuits due to saturation risks.

Perspectives on effective sample sharing Dr. Zhao raised the topic about improving reproducibility through sample sharing. Dr. Segal shared his experience with the NCI Early Detection Research Network Biobank, which has established biomarker reference laboratories (BRLs). These center labs had experience in transforming innovative assays to CLIA-grade clinical tests and are responsible for creating reference materials to share with others when they request samples from the biorepository. If any sites in the network had developed a promising biomarker, they could also send to BRLs for validation and reproducibility tests. Dr. Furdui suggested that technologies developed should undergo side-by-side comparison with standard methods in large trials for final validation.

Dr. Foster proposed to reserve some samples for the purpose of bridging technologies. In other words, use samples to test each of the new methods and use that as a reference across different projects. He emphasizes the importance of reference standards. Although there are intrinsic differences across different samples, for example, depending on the disease stages, if the same assay were run across those samples, a common set of reference would emerge. Pooled samples with various assay results could be used in the APS consortium to improve the precision and accuracy of all assays. Dr. Furdui thinks that a U54-type of funding mechanism might help to achieve this goal.

Dr. Ware mentioned the efforts by SCARAB and the APS consortium to balance high standards with practicality in sample sharing, advocating for federated databases and biorepositories. Dr. Zhao noted various existing NIH data repositories sorted by data types, and Dr. Ware highlighted the NHLBI BioDataCatalyst and BioLINK biorepositories. Dr. Foster shared his experience with BioLINK, noting the slow process, but there are available RFAs for sample utilization. As the resources under the Sepsis Biospecimens program grow, investigators have a unique opportunity to explore ways to share knowledge and samples efficiently to improve reproducibility and promote buy-in. Dr. Furdui added that a U54 mechanism would help with the IT component of the sample or data repositories.

Dr. Segal discussed centralized versus site-specific approaches for biobanks, emphasizing the need for an intentional framework including mechanisms for storage and sharing, regulatory compliance to allow sharing, and infrastructure to execute sharing. He noted the complexity of sharing involving contractual hurdles, even with NCI-facilitated agreements. Dr. Atreya agreed, urging collective efforts to address these barriers for future sharing.

Concluding Remarks

Meeting Co-Chairs:

Lorraine Ware, Ph.D., Michael Filbin, M.D., M.S.

Dr. Filbin concluded the meeting by saying that the fantastic discussion highlighted the strength of the collaboration as well as the difficulties of creating new biorepositories and sharing the samples. Dr. Ware thanks everyone for a great meeting. She is impressed with the great progress of the

program in the last 18 months. She learned a lot today about new methods and saw the sparks of potential new collaborations, which is exciting. She has a lot of ideas to take back to the APS consortium. Dr. Zhao thanked everyone for attending this meeting. She encourages everyone to move forward with their projects. As a follow-up to the Sepsis Biospecimens exploring the ways to use human samples, investigators can use the biospecimens in RPG projects for mechanistic studies, such as the high-risk, high-reward tech development program of the NIGMS, as well as the MIRA program of NIGMS. Dr. Chao thanked everyone for their informative presentations and thoughtful discussion. Dr. Furdui thanked the meeting organizers as well.