RNA Biosignatures: A Paradigm Change for the Management of Young Febrile Infants (Biosig II) PECARN Protocol Number 035

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PROTOCOL TITLE:

RNA Biosignatures: A Paradigm Change for the Management of Young Febrile Infants

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I confirm that I have read this protocol, I understand it, and I will conduct the study according to the protocol. I will also work consistently with the ethical principles that have their origin in the Declaration of Helsinki and will adhere to the Ethical and Regulatory Considerations as stated. I confirm that if I or any of my staff are members of the Institutional Review Board, we will abstain from voting on this protocol, its future renewals, and its future amendments.

Principal Investigator Name: _____

Principal Investigator Signature: _____

Date: _____

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Abstract

Every year ≈ 500,000 febrile infants ≤ 60 days of age present to US emergency departments (ED). Six to 10% of these febrile infants will have invasive bacterial infections (bacteremia, urinary tract infections or meningitis). Current approaches for the evaluation of young febrile infants are suboptimal because they include frequent invasive procedures, overuse of empirical antibiotics and unnecessary hospitalizations, which can lead to iatrogenic complications and have substantial cost implications. Microbiologic cultures of body fluids (blood, urine, and cerebrospinal fluid [CSF]) for diagnosis of bacterial infections have important limitations, including

a high rate of false positive results, an unknown rate of false negative results, and 24 – 48 hour turnaround time. In part because of these limitations, current guidelines for evaluation of febrile infants are sub-optimal. These guidelines include, but are not limited to, performing lumbar punctures, empiric treatment with broad-spectrum antibiotics and hospitalization until culture results are available. Thus, there is a clear need for developing diagnostic strategies for the evaluation of these febrile infants which are less invasive, more accurate and more timely.

The study investigators have identified novel and distinct RNA expression patterns (RNA biosignatures) in febrile infants $\leq$ 60 days of age. This proof-of-concept work has demonstrated well-defined RNA biosignatures in infants with bacterial and non-bacterial infections. Febrile infants with bacterial infections have distinct RNA gene expression patterns characterized by over-expression of inflammatory genes, while interferon genes are consistently over-expressed in febrile infants without bacterial infections. This previous work has identified 66 classifier genes that discriminate febrile infants with bacterial infections with 87% accuracy, suggesting a potentially more accurate reference than microbiologic cultures.

The long-term objective of this research protocol is to investigate whole genome RNA expression profiles to define RNA biosignatures that allow precise diagnosis of isolated bacterial infections, isolated viral infections and bacterial-viral co-infections in febrile infants $\leq$ 60 days of age. Addressing the optimal evaluation and management of the febrile infant will assure that this vulnerable population of children have the chance to achieve their full potential for healthy and productive lives, free from disease or disability.

1 Study Summary

This is a prospective, multi-center, cross-sectional study of febrile infants $\leq$ 60 days of age who are being evaluated for bacterial infections in the ED. After obtaining informed consent from the guardian research personnel will collect 3 ml of whole blood (1 ml for RNA expression analysis; 1 ml for comprehensive viral studies; and 1 ml for procalcitonin analysis) and 1 nasopharyngeal (NP) swab for a comprehensive respiratory viral diagnosis. Additionally, in a selected group of febrile infants who are either hospitalized from the ED or those who return to the ED, research personnel will obtain a second 1 ml blood sample for sequential RNA biosignature analysis at 6 – 84 hours after the initial sample collection. Using an aliquot of blood from the same sample, RNA biosignatures will be validated on a novel PCR-based platform.

1.1 Specific Aims

This project has the following Specific Aims:

Specific Aim 1. Define the RNA biosignatures in febrile infants $\leq$ 60 days of age with *isolated* bacterial infections, *isolated* viral infections, and viral-bacterial *co-infection*.

Specific Aim 2. Demonstrate the stability of the RNA biosignatures to distinguish febrile infants $\leq$ 60 days of age with isolated bacterial infections, isolated viral infections, and viral-bacterial co-infections over a 6 – 84 hour time period.

Specific Aim 3. Validate the RNA biosignatures on a novel, PCR-based platform that has a rapid (2 – 4 hours) turnaround time.

Specific Aim 4. Validate a previously derived decision rule for prediction of bacterial infection that includes procalcitonin measurement.

1.2 Hypotheses

The hypotheses of this study are:

1. Young febrile infants with isolated bacterial infections will express RNA biosignatures that allow discrimination from infants with isolated viral infections and from those with viral-bacterial co-infections with $> 90\%$ accuracy.

2. RNA biosignatures will retain their diagnostic ability for discriminating febrile infants with isolated bacterial infections, isolated viral infections and viral-bacterial co-infections at two distinct time points over 6 – 84 hours with $> 90\%$ accuracy.

3. RNA biosignatures for febrile infants with isolated bacterial infections, isolated viral infections, and viral-bacterial co-infections can be validated using a novel rapid turnaround PCR-based platform.

1.3 Subject Eligibility, Accrual and Study Duration

This study will enroll up to 2,800 eligible febrile subjects over a four to five year period in order to have an adequate number of infections (50 infants with bacteremia, 50 infants with urinary tract infection (UTI), 50 infants with isolated viral infection, and 50 infants with viral-bacterial co-infection). This is based on estimated infection rates[1] amongst febrile infants (8.7% prevalence of isolated bacterial infections, 2% rate of bacteremia,

7.4% rate of UTI, and 0.3% rate of meningitis). In addition, 50 healthy control infants will be recruited from well-baby clinics and day-surgery units to serve as a reference for the microarray analyses.

Inclusion criteria are:

1. Age $\leq$ 60 days; AND
2. Febrile with a documented rectal temperature $\geq$ 38° C in the ED *OR* have a history of fever measured by any route at home or outside clinic within 24 hours of ED presentation; AND
3. Being evaluated for SBI with blood screening tests including, but not limited to, blood culture.

Exclusion criteria are:

1. Prematurity (less than 37 weeks gestational age); OR
2. Administration of antibiotics within 4 days of ED presentation; OR
3. Overwhelming clinical sepsis requiring emergent interventions such as intubation and/or vasopressors; OR
4. Presence of a major congenital anomaly; OR
5. Presence of inborn errors of metabolism; OR
6. Presence of congenital heart disease; OR
7. Presence of chronic lung disease; OR
8. Presence of a disease or medication that would affect the immune system; OR
9. Indwelling shunts or catheters; OR
10. Evidence of focal infections such as abscess or cellulitis (otitis media is *not* an exclusion criterion); OR
11. Patient previously enrolled in this study.

2 Background and Rationale

2.1 Significance

Febrile Infants and Risk for Invasive Bacterial Infection. Every year $\approx$ 500,000 infants $\leq$ 60 days of age present to United States emergency departments (EDs) with fever as their presenting complaint.[2–4] Six to 10% of these febrile infants will have invasive bacterial infections (defined here as bacteremia, urinary tract infections [UTI], bacterial meningitis), henceforth referred to as "bacterial infections".[5, 6] During the first 2 months

of life, the infant's immune system is relatively immature. Chemotactic responses such as opsonin activity, macrophage function, and neutrophil activity are decreased, thus, making the infant more susceptible to bacterial infection.[7]

Current Approaches for the Evaluation of Febrile Infants ≤ 60 Days of Age. Clinical illness indicators are unreliable predictors of bacterial infection and as many as 65% of febrile infants with bacterial infections appear well on initial examination.[6, 8] Because there is no single reliable clinical or laboratory predictor of bacterial infections, clinicians currently depend on clinical guidelines for evaluation and management of febrile infants.[6, 7, 9, 10] These guidelines require varying combinations of blood, urine and cerebrospinal fluid (CSF) testing, and the latter requires performance of lumbar punctures (an invasive and painful procedure). Nearly 50 – 75% of the 500,000 infants will undergo clinical investigations, receive empiric broad spectrum antibiotics and will be hospitalized.[2–4]

Controversies in the Evaluation of Febrile Infants ≤ 60 Days of Age. Current approaches for the evaluation of young febrile infants are suboptimal. Many past studies were conducted on small cohorts of infants and used non-prospective study designs. Studies have used varying inclusion/exclusion criteria and may have been subject to enrollment and ascertainment bias. There is even substantial disagreement between infectious disease specialists regarding the definition of pathogens and contaminants,[11, 12] and the rate of false negative cultures is difficult to ascertain.[13] The need for performing routine lumbar punctures in well-appearing febrile infants has also been controversial.[14]

The primary reason for controversy is the lack of highly accurate, and short turnaround testing to reliably distinguish young febrile infants with and without bacterial infections.[15] Most importantly, there are limitations in the reference standard (microbiology cultures from clinically relevant fluid samples for the diagnosis of bacterial infections).[15, 16] Traditional culture techniques of blood, urine and CSF do not provide results quickly enough for ED disposition decisions because cultures require 24–48 hours turnaround time.[15–18] Many pathogens grow slowly or require complex media, limiting the impact on ED clinical decision-making.[15, 16, 18] Perhaps most importantly, 90% of all blood cultures do not grow any organism, and of the approximately 10% that do grow organisms, 50% represent true bacteremia (i.e. true positives) and 50% are contaminants (i.e. false positives). The latter are known to increase both the duration and costs of care.[14, 17, 19–25] Finally, a substantial number of clinically important pathogens remain unrecognized as they are resistant to cultivation in the laboratory (i.e. false negatives).[15, 16, 18, 26] In summary, there is a clear need for developing new, precise diagnostic tools that allow for less invasive,

more accurate, and more timely evaluation and diagnosis of young febrile infants in the ED, which is the ultimate goal of this study protocol.

2.2 Innovation

The primary innovation of this project is to examine the host response (patient's immune response) to bacterial and viral organisms, using RNA microarrays, and identify biosignatures that correctly classify the infants into bacterial, viral, co-infection, or uninfected groups. Since raw microarray results are not clinically friendly and require significant time, a modular PCR-based methodology will be used to bring this new diagnostic approach to the bedside, with sufficiently rapid turnaround to affect clinical decision making in the ED. Figure 1 provides an overview of the approach.

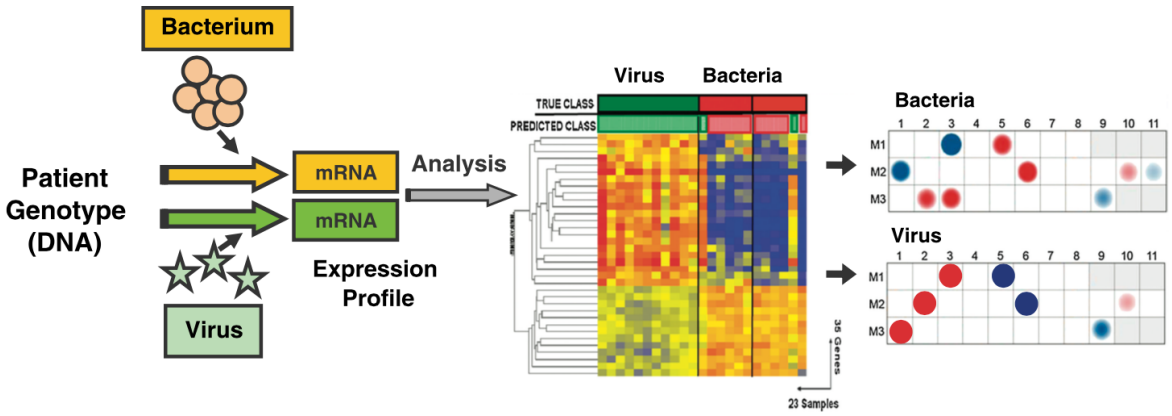

Figure 1: Microarray technology measures gene expression in response to infection (left panel), and patterns of gene expression are commonly shown as heat maps (middle panel). The modular analysis (right panel) summarizes differences in gene expression in a clinically friendly manner.

Host Response to the Presence of Pathogens. Many investigators have previously proposed alternative diagnostic approaches based on a comprehensive analysis of the host response to microbial pathogens.[1, 17, 26–29] Different microbial pathogens induce specific host responses that can be identified using RNA microarray assays in blood leukocytes.[28] Patterns of gene expression are known as "transcriptional signatures" or "RNA biosignatures."[1, 15, 26–29] This strategy shifts the paradigm in the diagnosis of infectious diseases from pursuing the identification of the pathogen to examining the host response as a unique disease biosignature. This approach can identify the etiology of infection indirectly and accurately.[1, 15, 28, 29]

Bacterial and Non-Bacterial RNA Biosignatures in Febrile Infants. Study investigators have studied host immune responses to infections in order to understand the complex pathogenesis of bacterial and viral illnesses and have defined RNA biosignatures in a variety of clinical conditions.[1, 27–29] Over the past decade, studies have been conducted in the Pediatric Emergency Care Applied Research Network (PECARN) to identify diagnostic RNA biosignatures for the evaluation of febrile infants.[1, 30–32] Results from these PECARN studies have established the potential that host-response molecular-based approaches can be used to accurately diagnose young febrile infants with bacterial and non-bacterial infections in the ED.[1] The current study will apply RNA biosignatures simultaneously with comprehensive viral testing. This will allow potential discrimination of febrile infants with isolated bacterial infections from those with isolated viral infections from those with viral-bacterial co-infections, and is a highly innovative approach with substantial clinical implications.

Shorter Turnaround Testing. This study will also evaluate a novel modular assay to expedite biosignatures-based diagnosis in the ED, a highly innovative approach that has the potential to fundamentally alter the evaluation and treatment of febrile infants. Indeed, RNA biosignatures may have superior performance characteristics when compared to the current microbiologic approach (cultures). This study offers a unique opportunity to change the paradigm of how febrile infants are evaluated in the ED.

In summary, the most critical and innovative contribution of this study is to bring RNA biosignatures to the point of care in clinical practice, in order to identify febrile infants who need early treatment and hospitalization, while avoiding unnecessary invasive testing, over-treatment, and unnecessary hospitalizations in febrile infants who do not require such interventions. In addition, the study may provide a better understanding of the value and limitations of current screening tests for bacterial infections. This will impact $\approx$ 500,000 young febrile infants annually in the United States, with substantial impact on quality and cost of care.

2.3 Preliminary Data

Distinct RNA Biosignatures. Febrile infants $\leq$ 60 days of age demonstrate measurable biosignatures in response to viral and bacterial pathogens. We have identified 3,690 differentially expressed transcripts among the 3 groups (infants with bacterial infections; non-bacterial infections and healthy controls). As shown in Figure 2 on the facing page, young febrile infants display distinct RNA biosignatures in the blood that are clearly different than the RNA biosignatures of healthy afebrile controls. These initial results

demonstrate that despite the immature immune system and short durations of illness, young infants display RNA biosignatures that can be exploited for diagnostic purposes.

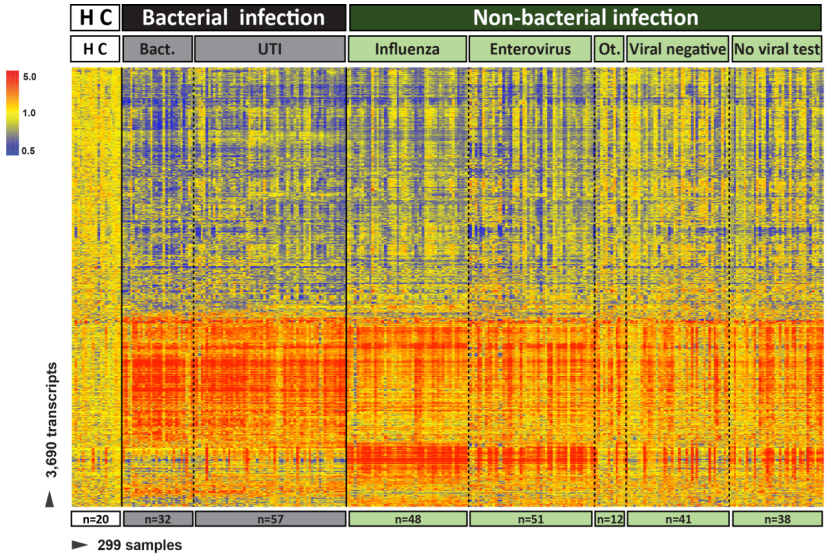

Figure 2: Febrile infants with bacterial and non-bacterial infections display distinct RNA biosignatures. Gene expression levels in health controls (HC) are used as reference and are shown in yellow; overexpressed transcripts are shown in red and under-expressed transcripts in blue.

Classifier Genes Discriminate Bacterial Infections. RNA biosignatures can discriminate infants with bacterial vs. non-bacterial infections, as shown in Figure 3 on the next page. Using the K Nearest Neighbor (KNN) algorithm, 66 classifier genes were identified in the training set (Figure 3 on the following page, left panel). These genes discriminated bacterial (n=44) from non-bacterial infections (n=95) with 82% sensitivity and 88% specificity. Validation of these genes in an independent test set (n=45 bacterial and 95 non-bacterial infections) confirmed 87% sensitivity and 88% specificity (Figure 3 on the next page, right panel)).

Interferon and Inflammation Responses. A novel modular analytical strategy can be used to characterize the biological significance of the bacterial and non-bacterial infection biosignatures.[27, 33–37] This analytical tool enables easy identification of the biological function of differentially expressed genes and facilitates data interpretation. Furthermore, this method makes a 2 – 4 hour turnaround time feasible. The bacterial

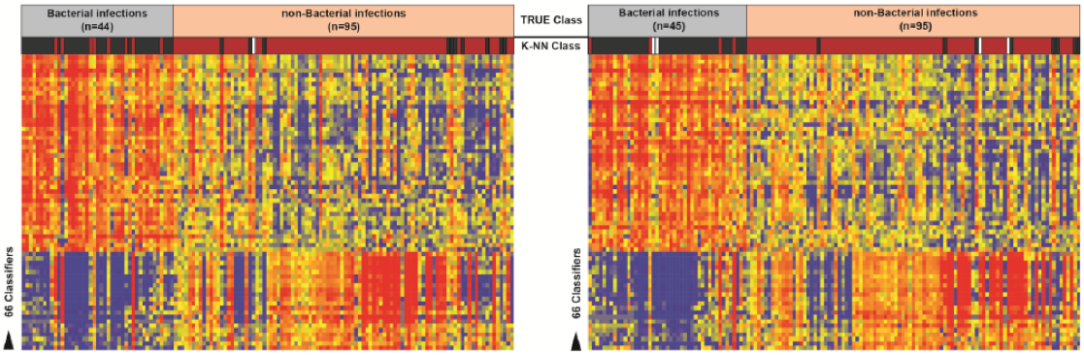

Figure 3: Classifier genes discriminate febrile infants with bacterial versus non-bacterial infections. 66 classifier genes were identified with the KNN algorithm in the training set (left panel), and validated in the test set (right panel).

infection group (Figure 4, left panel) shows significant over expression of inflammation modules and almost no over expression of interferon modules. Among the non-bacterial group, patients with influenza and enterovirus infections (Figure 4, right panel) show marked over expression of interferon modules and mild over expression of inflammation modules. Modular analysis also discriminates true bacteremia from false positive blood cultures for *Streptococcus viridans* (data not shown).

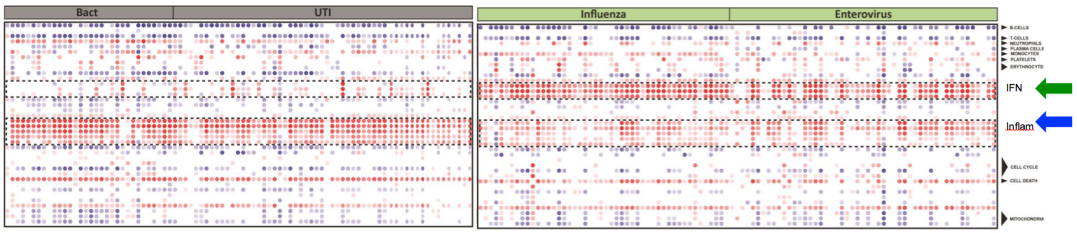

Figure 4: Modular analysis demonstrates the differences in expression of interferon and inflammation genes in bacterial versus non-bacterial infections. Inflammation modules are shown by the blue arrow, and interferon modules by the green arrow.

2.4 Rationale for Study

Despite the substantial initial achievements described in Section 2.3, several important steps remain before this technology can reach its full potential for use in the ED setting for the diagnosis of febrile infants.

Comprehensive Viral Testing. The detection of viral infections decreases the probability of, but does not eliminate the risk of bacterial infections in young febrile infants.[20] In our previous studies, 10% of febrile infants in the bacterial infection group demonstrated a simultaneous expression of inflammatory and interferon genes suggesting the presence of viral-bacterial co-infections.[1] Another 10 – 20% of febrile infants in the non-bacterial group who were not tested for viruses showed over-expression of interferon genes suggesting the presence of undetected viral infections.[29] In our previous study we intentionally chose to define bacterial RNA biosignatures and non-bacterial RNA biosignatures, and did not include comprehensive viral testing. In this study, comprehensive viral testing will be done for all subjects.

Temporal Stability of RNA Biosignatures. RNA biosignatures represent a "snap shot" of gene expression during the course of illness, but the duration of illness of febrile infants is usually not known. Data on duration of fever is subjective and unreliable, and furthermore, onset of fever does not correspond to activation of host response.[38] Host immune response is dynamic and changes over time.[39, 40] It is important to study temporal stability or changes of the RNA biosignature over the course of the disease and critical to document that the RNA biosignatures will retain discriminatory ability over a period of time. In this study, RNA biosignatures will be assessed on sequential blood samples at two defined time points. The impact of antipyretic medications and antibiotic administration on the RNA biosignatures must also be established.

Validation on a Novel PCR Platform. The defined bacterial, viral, and viral-bacterial co-infection RNA biosignatures will be validated on an independent set of febrile infants using a separate genomic platform. This PCR-based platform is currently being optimized and has substantially faster turnaround time (2 – 4 hours) than the current Illumina microarray chips. This platform has the potential of bringing this technology closer to clinical application in the ED.

3 Study Design and Procedures

3.1 Study Design Overview

This is a prospective, multi-center cross sectional study of febrile infants $\leq$ 60 days of age who are being evaluated for bacterial infections in PECARN emergency departments (ED). In order to have sufficient events (bacterial infections), up to 2,800 infants will be accrued over a four to five year period.

3.2 Participant Screening and Consent

PECARN research coordinators and investigators will identify febrile infants who are being evaluated for bacterial infection. Parental permission will be obtained.

3.3 ED Data and Specimen Collection

After parental permission has been obtained, the ED physician will determine the Yale Observation Score (YOS) and a minimal set of patient-related information. Each site will collect standard laboratory tests that include (but are not limited to): complete blood cell counts and differentials (CBC), urinalysis, CSF cell count, glucose and protein; bacterial cultures of blood, urine, CSF and stool. In addition, each site will perform their routine viral diagnostic tests according to their local standard of care. All these tests are obtained to allow the site to perform routine patient management, which will not be affected by this study. Final results of all these tests will be obtained from the clinical laboratory or the patient's medical record by PECARN research staff.

For study purposes only, the following biologic samples will be obtained in the ED. Results of analyses of these samples will not be available to the clinical team (as these analyses will be performed in batches at a central laboratory), and the costs of these analyses will be borne by the study budget. It is recognized that blood sampling can be difficult in infants. The priority is blood for clinical care first (cultures, CBC) followed by blood for RNA, followed by blood for viral testing, followed by blood for procalcitonin measurement.

Blood for RNA. RNA samples will be collected in appropriate tubes (up to 1 mL blood in baby Tempus tubes), placed in frozen storage (between -20 ° C and -80 ° C), and batch shipped to the Microarray Core at the central laboratory.

Blood for Viral Testing. Up to One mL blood will be collected in appropriate tubes (EDTA). Sample should be gently inverted 3 times, and placed UPRIGHT in frozen storage (between -20 ° C and -80 ° C), and batch shipped to the central laboratory for PCR testing for viruses. The initial testing will include enterovirus, parechovirus, adenovirus, and herpes simplex; additional viruses may be added in the future as new assays become available.

Respiratory Viral Testing. Nasopharyngeal (NP) swabs will be collected at the time of evaluation in the ED. Swabs will be placed in viral transport media, placed UPRIGHT

in frozen storage (between -20 ° C and -80 ° C), and batch shipped to the central laboratory for molecular testing. The respiratory viral panel will include influenza A and B, RSV, parainfluenza (1,2,3,4), metapneumovirus, adenovirus, rhinovirus/enterovirus, and coronaviruses.

Procalcitonin Measurement. Up to One mL blood will be collected in appropriate tubes; Sample will be processed accordingly, placed UPRIGHT in frozen storage (between -20 ° C and -80 ° C), and batch shipped to the central laboratory for measurement of procalcitonin.

3.4 Additional Specimen Collection

In febrile infants who are admitted to hospital, or among infants who are discharged home from the ED but return for re-evaluation, subsequent laboratory testing may be indicated. In those patients who meet clinical criteria for obtaining a second set of blood tests in that evaluation, a second 1 mL blood sample will be obtained for sequential RNA biosignature analysis. Results of a CBC should also be obtained at this time.

3.4.1 Compensation for Additional Specimen

Financial compensation may be provided to the research participant's legally authorized representative for the additional specimen collection attempts. Each site's Institutional Review Board must approve this compensation.

3.5 Healthy Control Cohort

A cohort of healthy, afebrile infants will be enrolled to serve as a reference for the microarray analyses. Control infants will be recruited in well-baby clinics and day-surgery units in a subset of the larger participating PECARN centers. The same specimens will be obtained from these infants as described for febrile infants in the ED setting (1 mL blood for RNA, 1 mL blood for PCR viral testing, and an NP swab for respiratory viral testing), except no sample will be obtained for procalcitonin. In addition, 1 mL will be sampled for a CBC.

3.6 Missed Eligible Febrile Infants

To assure that infants recruited into this study are representative of the overall febrile infant population, select screening, demographic, clinical and laboratory data will be col-

lected on missed eligible infants during year one. Waiver of informed consent is requested for this data collection.

At the end of the first year, the characteristics of the missed eligible infants will be compared with enrolled infants. If there are significant differences, then data collection will continue in subsequent years.

4 Data Elements

4.1 Screening and Eligibility

Patients who meet inclusion criteria will be entered into the database; exclusion criteria will be entered as well. For patients who meet inclusion and exclusion criteria (eligible for enrollment), we will record whether the parents are approached and consent obtained.

4.2 Demographics

The birthdate, gender, race and ethnicity of the patient will be collected. In addition, for those sites participating in the PECARN Data Registry Project, the medical record number and visit / encounter number will be recorded to enable potential linkage.

4.3 Clinical Data

Clinical characteristics of the infant's febrile illness will be collected. All components of the Yale Observation Scale will be collected. Antibiotic, antipyretic, and antiviral administration will be recorded. Parent and physician perception of the risk of infection will be recorded, as well as several questions about parental preferences for management of their infant.

4.4 Laboratory Data

All laboratory tests obtained during the ED visit are to be abstracted and entered into the study database. For infants who are admitted to the hospital, all cultures (i.e. blood, urine, CSF, stool), CSF tests, and viral studies obtained up to 7 days or through hospitalization are also to be abstracted and recorded.

The initial (and within 12 hours of sequential sample) CBC with differential and platelet counts will be recorded into the electronic data capture system (EDC), and the laboratory report should be uploaded as a file; this document should be de-identified

and a study ID should be added to it. Other blood tests that should be recorded, if obtained as part of clinical care, are C-reactive protein, procalcitonin (obtained for clinical care, not the study specimen). Urinalysis results should be entered into the EDC and the laboratory report should be uploaded as a de-identified file with a study ID. Chest radiograph (CXR) results should be recorded and for positive or equivocal studies, the de-identified report should be uploaded. CSF tests should be recorded in the EDC and laboratory report forms should be uploaded.

For all culture tests and viral studies, the reports will be uploaded unless the testing is completely negative. As with other uploads, the form should be de-identified and a study ID attached.

4.5 Follow Up

Follow up will be done for all infants who are discharged home from the emergency department and do not return within 72 hours, in order to confirm that they have not been subsequently diagnosed with a serious bacterial infection or viral infection, deteriorated clinically, or otherwise have evidence of infection. This follow up will include characteristics of any clinical care required since the infant was discharged from the hospital. Contact for follow-up will occur 7-14 days after the index ED visit. If contact is unable to be made within this timeframe, the patient's chart will be reviewed to obtain this information.

4.6 Specimen Handling Data

Information about specimen sampling, processing (when applicable), and freezer storage will be obtained. Shipping will be done in batches, and information will be collected for date and time shipped and received by the relevant central laboratory.

5 Data Analysis

The hypotheses of this study are:

1. Young febrile infants with isolated bacterial infections will express RNA biosignatures that allow discrimination from infants with isolated viral infections and from those with viral-bacterial co-infections with $> 90\%$ accuracy.

2. RNA biosignatures will retain their diagnostic ability for discriminating febrile infants with isolated bacterial infections, isolated viral infections and viral-bacterial co-infections at two distinct time points over 6 – 84 hours with $> 90\%$ accuracy.

3. RNA biosignatures for febrile infants with isolated bacterial infections, isolated viral infections, and viral-bacterial co-infections can be validated using a novel rapid turnaround PCR-based platform.

5.1 Specific Aim Analyses

Specific Aim 1. Define the RNA biosignatures in febrile infants ≤ 60 days of age with *isolated* bacterial infections, *isolated* viral infections, and viral-bacterial *co-infection*.

Blood samples for microarray analyses will be collected in Tempus tubes that allow recovery of high quality RNA from small blood volumes (1 mL) as required for studies in this infant population. After collection, samples will be stored at -20° C. Using state-of-the-art microarray software (GeneSpring, R, JMP/Genomics and Ingenuity Pathway Analysis), the following analyses will be carried out:

Unsupervised analyses. The objective is to group samples that pass quality control filters based on their molecular profile independently of their phenotypic classification (unbiased) and includes hierarchical clustering and principal component analysis (PCA).

Supervised analysis. The objective is to identify transcripts that are differentially expressed between study groups. Here, a priori knowledge of the phenotypic class is used to define the training and validation (test) sets.

Class comparisons. Comparative analyses between predefined sample groups will be performedusing Mann-Whitney or Kruskal-Wallis test for comparisons of $\geq$ two study groups, using a Benjamini-Hochberg false discovery rate (FDR) of 1% to correct for multiple testing and a ≥ 1.5 fold change filter in expression level relative to the control group.[41, 42]

Class prediction. K-Nearest Neighbors (K-NN) or support vector machine (SVM) algorithms will be used to identify the top ranked genes that best discriminate infants with bacterial, viral and bacterial-viral co-infections.[28, 36]

Module based analyses. Modules are groups of genes that share a similar function. To facilitate data interpretation and characterize the biological significance of the viral, bacterial and viral-bacterial signatures, we will apply this analytical framework.[34, 43] We have successfully leveraged this modular analytic strategy for diagnosis of multiple types of bacterial and viral infections.[33, 36, 44]

Molecular distance to health (MDTH). MDTH represents a tool that converts the global transcriptional perturbation of each sample into an objective molecular score that then can be correlated with clinical indices of severity as shown in our published studies.[36, 44–46]

To compare differences in transcriptional profiles across time, gene expression values will be $\log_2$ transformed and analyzed using linear mixed models for the viral, bacterial and viral-bacterial cohorts separately. Specifically, time will be included in the model as a categorical variable with a spatial power covariance matrix to account for correlation due to subjects having repeated measures. The 6–84 hour time point will be then compared to the baseline.[47, 48]

Specific Aim 2. Demonstrate the stability of the RNA biosignatures to distinguish febrile infants $\leq$ 60 days of age with isolated bacterial infections, isolated viral infections, and viral-bacterial co-infections over a 6 – 84 hour time period.

The RNA biosignature for the 3 groups will be compared between the initial sample (time 0) and the second sample (time 6 – 84 hours) to assess its temporal stability. In a second phase the ability of the second samples to discriminate among the different groups, following a similar approach with KNN and SVM algorithms, will be assessed. The linear mixed model produced in Specific Aim One will be applied using both the acute and second sample from infants in each of the categories (bacterial, viral and co-infections) separately, including time in the model as a categorical variable with a spatial power covariance matrix to account for correlation due to subjects having repeated measures.

Specific Aim 3. Validate the RNA biosignatures on a novel, PCR-based platform that has a rapid (2 – 4 hours) turnaround time.

Using the PCR-based assay to validate the biosignatures defined with Illumina microarrays, the specificity and sensitivity of the PCR based assay will be measured. RNA from subsets of febrile infants with bacterial, viral, and co-infections will be used. The top classifier genes that best discriminate between isolated bacterial infections, isolated viral infections and viral-bacterial co-infections (as identified using the Illumina platform) will

be adapted to the PCR platform which can accommodate up to 90-100 transcripts:

- The analyses on the PCR platform will be run on a total of 75 samples; n=25 from each category, including bacterial infections, viral infections, and bacterial-viral co-infections. We will compare the results obtained from the PCR-based and Illumina platforms. Once we confirm similar (or improved) sensitivity and specificity, we will continue to the next validation step.

- We will then validate the diagnostic biosignatures directly on the PCR platform using an independent group of samples (n=125). For the comparative analysis between the test accuracies using the Illumina vs. the PCR platforms we will use traditional statistical methods to measure sensitivity and specificity and compared with both the reference standard (cultures and viral PCR tests) and Illumina-based biosignatures.

Specific Aim 4. Validate a previously derived decision rule for prediction of bacterial infection that includes procalcitonin measurement.

The investigators will perform a validation of the previously derived prediction rule developed for identification of infants with serious bacterial infections. The analysis will adhere to established prediction rule methods and STAndards for the Reporting of Diagnostic accuracy studies (STARD) guidelines. Three predictors were identified in the rule (positive urinalysis, ANC $> 4000/mm^3$, and PCT > 0.5 ng/ml). Absence of these would identify an infant as having very low risk of SBI. Rule performance will now be evaluated in the new cohort of infants enrolled in the current study. We will report test characteristics in the validation group and calculate 95% confidence intervals (CI) with exact methods.

5.2 Sample Size Calculations and Statistical Power

The primary goal is to define RNA biosignatures in the following subgroups of febrile infants:

- Isolated Bacterial Infections Group: This group includes febrile infants with bacteremia, bacterial meningitis, and UTIs.
- Isolated Viral Infections Group: This group includes febrile infants with negative bacterial cultures of the blood, CSF, and urine in association with a positive viral study.

- Viral-Bacterial Co-Infections Group: This group includes febrile infants with simultaneous positive bacterial cultures (urine, blood, or CSF) and a positive viral study.
- Viral and Bacterial Negative Group: This group includes febrile infants with negative bacterial cultures of the blood, CSF and urine and negative results from comprehensive viral testing.

The sample size required in each of the above mentioned categories is based on achieving an accuracy of >90% for the RNA candidate biosignatures. It is necessary to recruit at least 50 subjects in each of the first three groups, as well as 50 health afebrile infants for controls. This allows for a training set of 75 subjects (25 per group) that will be validated in a new cohort of 75 subjects [test setbacterial (n=25), viral (n=25), bacterial-viral (n=25)]. With these numbers of events, the study is adequately powered (>90%) for the detection of at least 658 true differentially expressed genes between infants with bacterial, viral, and bacterial-viral co-infections. Power calculations were based on conservative estimates obtained from the preliminary microarray data (two-sided t-test, fold change of 1.5 SD of $\pm$ 0.3, and false discovery rate (FDR) of 1% for the typically 18,000 genes present in at least one sample). In addition, the study is adequately powered to define the RNA biosignatures for the isolated bacterial, isolated viral, and viral-bacterial co-infections biosignatures groups with an estimated accuracy of $>$ 90%.

6 Data Management

6.1 Clinical Site Data Management

The Data Coordinating Center will create the electronic data capture (EDC) system and worksheets that can be used by clinical site research coordinators and investigators. Data will be entered via the Web into the EDC. Worksheets and study documents will be maintained in locked filing cabinets in locked offices at each site.

6.2 Electronic Data Capture System

The Data Coordinating Center currently uses OpenClinica as its data capture system; this may be changed at any time without requiring a protocol amendment.

6.3 Study Monitoring

The investigators recognize the importance of insuring data of excellent quality. Monitoring is critical to this process. Currently, PECARN studies have monitoring plans in place, coordinated by the DCC in conjunction with the study PIs. Monitoring has been a very effective tool for maintaining data quality in previous PECARN studies. The monitoring plan is designed to verify the site's compliance with the study methodology, data quality, patient protection and adherence to Good Clinical Practice guidelines and to identify problems with sites and methods for handling problems that arise. Each site will be monitored throughout active study enrollment. A number of methods may be employed to assure quality data in this study including: site monitoring, remote monitoring and database monitoring. A supplemental study-specific monitoring plan, separate from the protocol, will be developed by the DCC prior to the start of the study, and will outline specific criteria for monitoring.

6.4 Data Coordinating Center

Data Center Description

The Data Coordinating Center (DCC) in the Department of Pediatrics at the University of Utah School of Medicine provides data coordination and management services for a variety of national research networks. Anchoring these services is a new state-of-the-art, energy efficient data center completed in 2013. The data center facility supports more than 1200 users around the world and provides a secure, reliable, enterprise-wide infrastructure for delivering critical DCC systems and services. The new data center was built using high industry standards and energy efficient cooling solutions. The data center is cooled by Rittal's LCP inline cooling technology, providing efficiency, redundancy and modularity. Cooling is based upon a hot/cold aisle design that allows for even air distribution with minimal hot spots. The data center electrical power system contains a redundant Mitsubishi uninterruptible power system (UPS) with a diesel backup generator. The data center is protected with a FM200 fire suppression system, early warning smoke detectors and a heat detection warning system to act as a secondary system to the smoke detectors. Security guards are on-site conducting access control and rounds 24/7/365. Entry into the data center is restricted by card access and layered security measures and controls. The data center and external building access points are monitored with video surveillance.

In 2011 the data center began a large scale VMware server virtualization deployment. Currently, the data center has virtualized about 95% of its environment. The virtual

environment consists of more than 160 virtual servers and nearly 20 physical servers. The data center's virtualization solution provides key advantages:

- high availability – in the event of hardware failure, virtual servers automatically go back online in a seamless process.
- flexible infrastructure – disk storage, memory and processor capacity can be increased or reallocated at any time.
- rapid deployment – servers can be provisioned on-demand with minimal waiting on hardware of software.

The data center also enhanced its storage resources by implementing a networked storage system to support its virtualized environment. The data center currently manages over 50 terabytes of data. The storage solution consists of Dell's EqualLogic PS Series Storage system for providing a virtualized storage area network (SAN). Some of the benefits that are realized through this technology are:

- storage architecture is no longer be a bottleneck for IT services;
- performance is better than with the previous architecture;
- tiered storage is now possible;
- provisioning and reclamation of SAN disk will be much easier; and most important,
- the new architecture includes a redesign of the SAN fabric to include complete redundancy.

Production servers running critical applications are clustered and configured for failover events. Servers are backed up with encryption through a dedicated backup server that connects across an internal 10 gigabit network to a tape drive. DCC storage area networking (SAN) applications, clusters, and switch-to-switch links are also on a 10 gigabit network. Incremental backups occur hourly Monday through Friday from 6 am to 6 pm. Incremental backups also are performed each night with full system backups occurring every Friday. Tapes are stored in a fireproof safe inside the data center facility, and full backups are taken off site on a weekly basis to an off-site commercial storage facility.

In the event of catastrophic failure, such as a fire in the server facility, daily backups would probably survive because of the fire suppression system and fireproof safe, but there would be obvious delay in re-establishing data center function because the servers will not survive such a disaster. Total destruction of the data center facility could cause the loss of up to one week's data. In future investments, the data center is making co-location, disaster recovery and business continuity solutions a top priority.

DCC information systems are available 24 hours a day, 7 days a week to all users unless a scheduled maintenance interruption is required. If this occurs, we notify all users of the relevant systems, and data entry can be deferred until after the interruption is over. Critical systems availability has exceeded 99.9% for the past two years, and there has been no unscheduled downtime in over five years.

Security and Confidentiality

The data center coordinates the network infrastructure and security with the Health Sciences Campus (HSC) information systems at the University of Utah. This provides us with effective firewall hardware, automatic network intrusion detection, and the expertise of dedicated security experts working at the University. Network equipment includes four high-speed switches. User authentication is centralized with two Windows 2008 domain servers. Communication over public networks is encrypted with virtual point-to-point sessions using secure socket layer (SSL) or virtual private network (VPN) technologies, both of which provide at least 128 bit encryption. All of our Web-based systems use the SSL protocol to transmit data securely over the Internet. Direct access to data center machines is only available while physically located inside our offices, or via a VPN client.

All network traffic is monitored for intrusion attempts, security scans are regularly run against our servers, and our IT staff is notified of intrusion alerts. Security is maintained with Windows 2008 user/group domain-level security. Users are required to change their passwords every 90 days, and workstations time out after 5 minutes of inactivity. All files are protected at group and user levels; database security is handled in a similar manner with group-level access to databases, tables, and views in Microsoft SQL Server. Finally, all laptop computers in use in the DCC or in the Department of Pediatrics are whole-disk encrypted.

The data center uses control center tools to continuously monitor systems and failure alerts. Environmental and network systems are also monitored to ensure up time. Highly trained system administrators on staff are available to respond in high risk emergency events.

All personnel involved with the DCC have signed confidentiality agreements concerning data encountered in the course of their daily work. All personnel (including administrative staff) have received Human Subjects Protection and Health Information Portability and Accountability Act (HIPAA) education. We require all users to sign specific agreements

concerning security, confidentiality, and use of our information systems, before access is provided.

6.5 Record Access

The medical record and study files (including informed consent, permission, and assent documents) must be made available to authorized representatives of the Data Coordinating Center, upon request, for source verification of study documentation. In addition, medical information and data generated by this study must be available for inspection upon request by representatives (when applicable) of the Food and Drug Administration (FDA), NIH, other Federal funders, and the Institutional Review Board (IRB) for each study site.

7 Protection of Human Subjects

7.1 Institutional Review Board (IRB) Approval

The Data Coordinating Center and each clinical center must obtain approval from their respective IRB prior to participating in the study. The Data Coordinating Center will track IRB approval status at all participating centers and will not permit subject enrollment without documentation of initial IRB approval and maintenance of that approval throughout subsequent years of the project.

7.2 Informed Consent

Parental Permission

Subjects who are eligible for this study are infants, and written permission from parents or legal guardians will be required for participation. After determining that a subject is eligible, the site investigator or designee will approach the parent or legal guardian to offer participation for their child in the study The parent or legal guardian will be informed about the objectives of the study and the potential risks and benefits of participation. Whether the parent or legal guardian provides or refuses permission for their infant to participate, all clinical management will be provided by the clinical staff in accordance with institutional practice and judgment.

7.3 Potential Risks

There are no major risks associated with participating in this study. Risks relate to venipuncture and collection of clinical data. Blood samples will be carried out during

a venipuncture that is being done as part of clinical care, or may also be done with a separate venipuncture solely for purposes of the research protocol. Possible side effects of venipuncture include pain, bruising, bleeding, or infection at the blood drawing site. The total volume of blood obtained for this study in the ED will not exceed 3 mL, and second samples obtained in hospitalized infants will be 1 mL.

7.4 Protections Against Potential Risks

The clinical sites are expert in the management and care of febrile infants in the emergency department and venipuncture will be carried out by qualified clinical personnel. The risk of disclosure of confidential information is minimized by the security systems described for the Data Coordinating Center (Section 6.4).

7.5 Potential Benefits

There are no immediate potential benefits for subjects who participate in this study. The results of study samples used for comprehensive viral testing (whole blood PCR and NP swab), microarray analyses, and procalcitonin measurements will not be available during the management of the infant, and will not be available to the clinical site.

7.6 Importance of Knowledge to be Gained

Development of robust bacterial, viral, co-bacterial-viral and non-bacterial biosignatures confers potential substantial benefits to infants who present to EDs and/or to their primary care providers office for evaluation of fever in the future. Biosignatures that have been validated on a large cohort of febrile infants from diverse backgrounds and that can discriminate between those who have bacterial infections versus those who do not have bacterial infections have great potential for reducing infant morbidity and mortality. Potential benefits include greater accuracy in early identification of bacterial infections, reduction in the need for empiric invasive testing including lumbar punctures if the transcriptional biosignatures prove to be sufficiently accurate, reduction in the use of antibiotics for patients identified to have non-bacterial infections, reduction in the need for hospitalization pending identification of bacterial pathogen, and a potential reduction in the overall costs associated with evaluation and management of the febrile infant if other laboratory testing is obviated. We anticipate, if successful, findings from this study will impact the evaluation of $\approx$ 500,000 febrile infants every year in the United States EDs.

8 Study Training

8.1 Study Training

A formal training program for investigators and research staff will be held prior to the start of enrollment. The training program will cover regulatory topics and Good Clinical Practice. The training will also provide in depth explanations regarding study procedures, laboratory sample handling, data entry procedures, quality assurance, site monitoring, and the informed consent process. A manual of operations will be provided to each investigator prior to the start of enrollment. The manual will detail specific information about the study procedures, regulatory information, safety reporting, and other necessary information. Updates and revisions to the manual will be made available electronically. The Data Coordinating Center, in collaboration with the study investigator (Dr. Mahajan, Ramilo, Kuppermann), will be the main contact for study questions.

9 Regulatory Issues

9.1 Health Insurance Portability and Accountability Act

Data elements collected include the date of birth and date of admission. Prior to statistical analyses, dates will be used to calculate patient age at the time of the study events. The final data sets (used for study analyses and archived at the end of the study) will be de–identified, and will exclude these specific dates.

Data elements for race, ethnicity, and gender are also being collected. These demographic data are required for Federal reporting purposes to delineate subject accrual by race, ethnicity, and gender.

For purposes of the DCC handling potential protected health information (PHI) and producing the de–identified research data sets that will be used for analyses, all study sites have been offered a Business Associate Agreement with the University of Utah. Copies of executed Business Associate Agreements are maintained at the DCC.

9.2 Inclusion of Women and Minorities

There will be no exclusion of patients based on gender, race, or ethnicity.

9.3 Retention of Records

For federally funded studies subject to the Common Rule, records relating to the research conducted shall be retained for at least 3 years after completion of the research. Completion of the research for this protocol should be anticipated to include planned primary and secondary analyses, as well as subsequent derivative analyses. Completion of the research also entails completion of all publications relating to the research. All records shall be accessible for inspection and copying by authorized representatives of the regulatory authorities at reasonable times and in a reasonable manner [45 CFR §46.115(b)].

9.4 Public Use Data Set

After subject enrollment and follow up have been completed, the DCC will prepare a final study database for analysis. A releasable database will be produced and completely de-identified in accordance with the definitions provided in the Health insurance Portability and Accountability Act (HIPAA). Namely, all identifiers specified in HIPAA will be recoded in a manner that will make it impossible to deduce or impute the specific identify of any patient. The database will not contain any institutional identifiers.

The DCC will also prepare a data dictionary that provides a concise definition of every data element included in the database. If specific data elements have idiosyncrasies that might affect interpretation or analysis, this will be discussed in the dictionary document. In accordance with policies determined by the investigators and funding sponsors, the releasable database will be provided to users in electronic form.

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