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Using Cluster Analysis of Cytokines to Identify Patterns of Inflammation in Hospitalized Patients with Community-acquired Pneumonia: A Pilot Study

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Erratum
In Table 1, the patient identification numbers, along with the heading for these, were inadvertently omitted. The numbers are important to match with those in the heatmaps and dendrograms in Fig. 1. The error has been corrected.

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Abstract

Purpose: Patients with severe community-acquired pneumonia (CAP) are believed to have an exaggerated inflammatory response to bacterial infection. Therapies aiming to modulate the inflammatory response have been largely unsuccessful, perhaps reflecting that CAP is a heterogeneous disorder that cannot be modulated by a single anti-inflammatory approach. We hypothesize that the host inflammatory response to pneumonia may be characterized by distinct cytokine patterns, which can be harnessed for personalized therapies.

Methods: Here, we use hierarchical cluster analysis of cytokines to examine if patterns of inflammatory response in 13 hospitalized patients with CAP can be defined. This was a secondary data analysis of the Community-Acquired Pneumonia Inflammatory Study Group (CAPISG) database. The following cytokines were measured in plasma and sputum on the day of admission: interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-6, CXCL8 (IL-8), IL-10, IL-12p40, IL-17, interferon (IFN)γ, tumor necrosis factor (TNF)α, and CXCL10 (IP-10). Hierarchical agglomerative clustering algorithms were used to evaluate clusters of patients within plasma and sputum cytokine determinations.

Results: A total of thirteen patients were included in this pilot study. Cluster analysis identified distinct inflammatory response patterns of cytokines in the plasma, sputum, and the ratio of plasma to sputum.

Conclusions: Inflammatory response patterns in plasma and sputum can be identified in hospitalized patients with CAP. Characterization of the local and systemic inflammatory response may help to better discriminate patients for enrollment into clinical trials of immunomodulatory therapies.

1 Introduction

Even with appropriate antibiotic therapy, some hospitalized patients with community-acquired pneumonia (CAP) progress to clinical failure and death. High cytokine levels produce an exaggerated inflammatory response, known as the cytokine storm, and may drive poor clinical outcomes. As a result, investigators have evaluated the use of immunomodulatory agents in an attempt to control this exaggerated inflammatory response and reduce patient mortality. Corticosteroids are the primary immunomodulatory agents considered for patients with CAP, and results of several investigations[1–10] have been evaluated in recent systematic reviews and meta-analyses[11,12] Although steroids are considered important immunomodulatory agents for these patients, other considerations include macrolides[13–18] statins[19–29] and tissue factor pathway inhibitor[30].

Severe pneumonia at the time of hospital admission is the primary criterion for patient enrollment into clinical trials of immunomodulatory therapies[10,31]. This is due to a general consensus that severity of disease and the inflammatory response are positively correlated. If this is the case, patients with more severe pneumonia should present with an exaggerated inflammatory response and would be ideal candidates for enrollment into trials of immunomodulatory therapies. Although this inflammation-severity correlation may be true at the population level, increased disease severity at the individual level may also be impacted by factors

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unrelated to the inflammatory response. For example, a patient hospitalized with CAP with a prior history of congestive heart failure who develops severe respiratory deterioration due to cardiogenic pulmonary edema may not have an exaggerated inflammatory response. Any patient without an exaggerated inflammatory response, regardless of disease severity, would not be an appropriate candidate for a trial of an immunomodulatory therapy. Several experts in the field have indicated that the failure of many recent investigations into immunomodulation in hospitalized patients with CAP is likely to be related to our inability to properly characterize the patients who would benefit from these therapies prior to enrollment[32,33]. To address this concern, methods to better identify patients with and without an exaggerated inflammatory response are needed. We hypothesize that patients with CAP can be clustered into distinct inflammatory response groups at the time of hospital admission using cytokine levels in plasma and/or sputum.

The objective of this study was to evaluate the utility of hierarchical agglomerative cluster analysis of plasma and sputum cytokines to examine if inflammatory patterns can be defined in hospitalized patients with CAP.

2 Methods

2.1 Study design and setting

This was a secondary data analysis of data previously collected from the Community-Acquired Pneumonia Inflammatory Study Group (CAPISG). An in-depth description of the CAPISG study and specimen collection has been previously published[34]. Briefly, we prospectively enrolled non-consecutive adult patients hospitalized with CAP at the University of Louisville Hospital, Louisville, Kentucky, USA, from 01 April 2011 to 01 August 2012. The study protocol required enrolled patients to provide blood and sputum specimens on the day of admission and at regular intervals during hospitalization.

2.2 Study population

Adult patients with CAP in whom both blood and sputum specimens were obtained on the day of admission were included in the current pilot study. CAP was defined as a new pulmonary infiltrate (within 24 hours of admission), associated with at least one of the following criteria: a new or increased cough, an abnormal temperature (<35.8 °C or >37.8 °C), or an abnormal leukocyte count (leukocytosis, leukopenia or the presence of immature neutrophils). Pneumonia was defined as community-acquired if a patient had no history of hospitalization during the 2 weeks prior to admission. Patients were excluded if they had a medical history that, in the investigator's opinion, precluded subject compliance with the protocol.

2.3 Data sources and measurements

After patient consent, trained study coordinators and/or research associates collected clinical data from the patient's medical record onto a paper case report form. A separate research associate entered these data into a secure, web-based data management sys-

tem hosted by the University of Louisville Division of Infectious Diseases. Data quality issues identified by the research associate entering the data were fixed prior to submission of the case to the database. The database also contains several data quality checks to limit out-of-range errors and inappropriate data types. These data quality structures were built in based on our decades of experience collecting and entering clinical data into electronic databases. After all data queries were resolved, the case was accepted into the database for analysis. The following clinical variables were collected and evaluated:

1. **Demographics:** age and sex

2. **Comorbid conditions:** chronic obstructive pulmonary disease (COPD), cerebrovascular disease, congestive heart failure, chronic renal disease, diabetes mellitus, active coronary artery disease, neoplastic disease, neurologic disease/mental illness, acute renal disease, hyperlipidemia, HIV status, ACE inhibitor use, aspirin use, beta blocker use, heparin use, statin use, hospitalization for 2 or more days in the prior 90 days, home infusion therapy, home oxygen therapy, nursing home resident, and atrial fibrillation.

3. **Physical examination:** oral temperature, heart rate, respiratory rate, systolic blood pressure, and diastolic blood pressure.

4. **Laboratory tests:** pH, FiO_2, $PaCO_2$, serum bicarbonate, serum blood urea nitrogen (BUN), serum creatinine, serum glucose, hematocrit, hemoglobin, serum potassium, serum sodium, platelet count, procalcitonin, and white blood cell count (WBC).

5. **Severity of CAP:** need for ICU admission on arrival, altered mental status, vasopressors, IV steroids on admission, and the Pneumonia Severity Index.

All microbiological evaluations were done according to clinical needs per hospital microbiology protocols.

2.4 Specimen collection and processing

2.4.1 Plasma

Blood samples were obtained on the day of admission to the hospital. Venous blood was collected using sodium citrate BD Vacutainer®tubes and processed within 2 hours of collection. The tubes were centrifuged at $300 \times g$ for 10 minutes and the plasma separated by aspiration, aliquoted (0.5 ml each) and stored frozen at -80 °C until assayed.

2.4.2 Sputum

Sputum samples were obtained on the day of admission to the hospital and processed within 2 hours of collection, according to the method described by Pizzichini et al.[35]. Briefly, samples were diluted with an equal amount of a 0.1% Dithiothreitol solution in phosphate buffered saline (PB and incubated in a rocking platform for 15 minutes in order to digest the mucus. An equal volume of sterile saline was added, followed by an additional 5 minutes of incubation. The samples were then filtered through

Table 1 Patient Characteristics

Variable	Patient Identification Number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Sex	Male	Female	Male	Female	Male	Male	Female	Female	Female	Male	Male	Male	Male
Age (years)	78	54	40	51	63	62	72	57	53	78	85	65	59
Obese (BMI>30 kg/M2)	No	Yes	No	Yes	No	No	Yes	Yes	No	No	No	No	No
Immunocompromized	No	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes
ICU admission	Yes	No	No	No	No	No	No	No	Yes	No	Yes	No	Yes
Nursing home resident	No	No	No	No	No	No	No	Yes	No	No	No	No	Yes
Active cancer	No	No	No	No	No	Yes	Yes	No	No	Yes	No	No	No
Liver disease	No	No	No	No	No	No	No	No	No	No	No	No	No
Renal disease	No	No	No	No	Yes	No	No	No	No	No	No	No	No
Congestive heart failure	No	No	No	Yes	No	No	Yes	Yes	No	Yes	No	No	No
Cerebrovascular disease	No	No	No	No	No	No	No	Yes	No	No	Yes	No	No
Altered mental status	No	No	No	No	No	No	No	No	No	Yes	No	No	No
Heart rate, (beats/min)	91	130	98	78	93	87	118	108	77	94	156	92	102
Respiratory rate breaths/min	18	22	24	16	22	20	28	24	28	20	40	25	25
Systolic blood pressure (mmHg)	95	144	114	135	99	115	187	166	185	133	99	151	90
Oral temperature (F)	99	100	100	98.7	100.4	98.6	102	101	101	99.2	100	97.5	98
Hematocrit (%)	41.4	48.8	38	40.3	42.5	35	34	43.5	36.1	31.8	43.2	34.2	37.7
Blood urea nitrogen mg/dl	13	16	13	22	36	40	10	31	5	70	21	10	13
Sodium mmol/litre	137	134	140	133	133	129	140	133	140	146	140	139	138
Arterial pH	No ABG	7.36	No ABG	7.46	No ABG	7.41	No ABG	7.39	7.44	No ABG	7.41	7.414	No ABG
PaO2 mmHg	No ABG	59	No ABG	60	No ABG	67	No ABG	94	66	No ABG	78.4	65.9	No ABG
Glucose mg/dl	116	97	101	295	88	102	105	185	107	108	179	111	174
Pleural effusion	No	Yes	No	No	No	No	No	Yes	No	No	Yes	No	Yes
Pneumonia Severity Index risk class	Class III	Class III	Class I	Class II	Class IV	Class V	Class IV	Class IV	Class II	Class V	Class V	Class II	Class III
Days to clinical stability	2	2	7	2	3	Unknown	4	6	4	2	6	2	3
Culture positive	Yes	Yes	Yes	Yes	No	Yes	No	No	No	No	No	Yes	No
Length of stay (days)	3	4	8	3	3	15	6	6	6	6	6	2	9
In-hospital mortality	No	No	No	No	No	Yes	No	No	No	No	No	No	Yes

nylon gauze and centrifuged at $790 \times g$ for 10 minutes. The cell-free supernatants were aliquoted and stored frozen at -80 °C until used for the measurement of cytokine levels.

2.5 Cytokine measurements

Laboratory methods for cytokine measurements have been previously published[34]. The concentrations of interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-6, CXCL8 (IL-8), IL-10, IL-12p40, IL-17, interferon (IFN)γ, tumor necrosis factor (TNF)α, and CXCL10 (IP-10) in plasma and sputum samples were determined using Milliplex MAP Multiplex kits (EMD Millipore, Billerica, MA). Following thawing, plasma and processed sputum samples were centrifuged at $10,000 \times g$ for 5 min and the supernatants used in the assay according to the manufacturer's instructions

2.6 Compliance with Ethical Standards

The University of Louisville Human Subjects Program Protection Office approved this study prior to any data collection (approval number: 07.0182). Informed consent was obtained from all individual participants included in the study.

2.7 Statistical analysis

For each patient, cytokine values were scaled by subtracting the mean cytokine value across all patients from each particular patient cytokine level. This value was then divided by the standard deviation of the particular cytokine. The scale function in Rv3.2.0 (R Foundation for Statistical Computing, Vienna, Austria) was used for scaling purposes. Hierarchical agglomerative clustering algorithms were then used to evaluate clusters of patients within the plasma and sputum determinations separately. Finally, the plasma determination was divided by the sputum determination and the same clustering methods were used to evaluate clusters from the plasma/sputum ratio. Ensemble cluster-

ing methods were used to identify the most appropriate distance metric and cluster linkage function for hierarchical agglomerative cluster analysis[36]. From these results, the distance metric and linkage function providing the most clinically meaningful clustering were chosen. For each clustering algorithm, dendrograms and heatmaps were created to visualize the clusters and scaled cytokine values. Dark blue colors on the heatmaps represent high cytokine values, white colors represent average values, and dark red represent low values. To compare plasma and sputum clustering dendrograms, we used the Pearson cophenetic correlation statistic. Briefly, cophenetic correlation statistics are similar to traditional correlation measures, ranging from -1 (perfect negative correlation) to +1 (perfect positive correlation), with a zero being no correlation.

To compare differences in patient characteristics between groups defined as having a higher inflammatory response versus a lower inflammatory response from the cluster analysis, frequencies and percentages for categorical variables and medians with interquartile ranges were presented for continuous variables. Statistical hypothesis testing was not done due to the probability of error in the multiple tests and the limited number of patients included in the study. R v3.2.0 (R Foundation for Statistical Computing, Austria) was used for all analyses. The following R packages and were used: gplots[37], dendextend[38], dendextendRcpp[39], clue[36], and RColorBrewer[40].

3 Results

3.1 Demographic, clinical and laboratory findings

A total of 13 patients were included in this pilot study. Baseline patient characteristics and clinical outcomes for each of the 13 patients can be found in **Table 1**. No patients were started on vasopressors on admission or during hospitalization. For higher vs lower inflammatory phenotype groups identified via cluster anal-
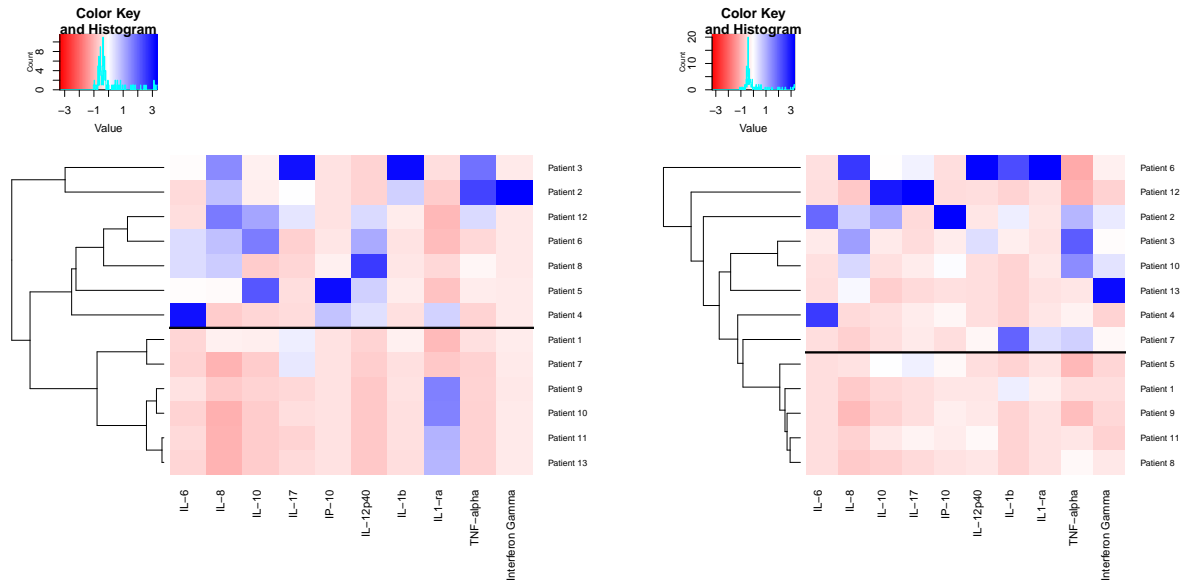
(a) sputum

(b) plasma
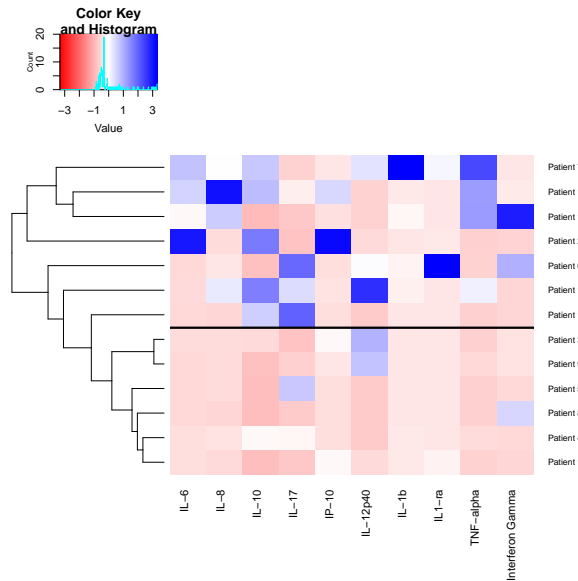
(c) plasma/sputum ratio

Fig. 1 Heatmaps and dendrograms for cytokines in sputum and plasma. Each heatmap and dendrogram depict some separation of patients' inflammatory response. The cophenetic correlation coefficient comparing the clustering of patients in plasma and sputum determinations was 0.14, suggesting low correlation between plasma and sputum clusterings. a horizontal line was drawn to indicate a suggested separation of inflammatory response patterns based on the cluster dendrograms and visual inspection of the heatmaps.

ysis of the sputum specimens patients with a higher inflammatory phenotype had a lower rate of ICU admission (0% vs 67%, respectively). Similarly, when evaluating plasma cytokine profiles, patients with a higher inflammatory phenotype had a lower rate of ICU admission versus those with a lower inflammatory phenotype (12% vs 60%, respectively). This difference did not remain when evaluating the plasma/sputum ratio. No other clinically meaningful differences were identified.

A pathogen was identified in 6 of the 13 patients. Streptococcus pneumoniae was identified in patients 1, 4, and 12, influenza in patient 2, Pseudomonas aeruginosa in patient 3, and methicillin-resistant Staphylococcus aureus in patient 6.

3.2 Cytokine profiles

Based on the results of ensemble clustering, Euclidean distance and Ward's D linkage metrics were identified as the most clinically meaningful for our needs. Each heatmap and dendrogram depict some separation of patients' inflammatory response. A heatmap and dendrogram for cytokines in sputum is depicted in **Figure 1a**. A heatmap and dendrogram for cytokines in plasma is depicted in **Figure 1b**. The cophenetic correlation coefficient comparing the clustering of patients in plasma and sputum determinations was 0.14, suggesting low correlation between plasma and sputum clusterings. A heatmap and dendrogram for cytokines in plasma/sputum ratio is depicted in **Figure 1c**. In each figure, a horizontal line was drawn to indicate a suggested separation of inflammatory response patterns based on the cluster dendrograms and visual inspection of the heatmaps.

4 Discussion

This pilot study suggests that it may be possible to identify distinct cytokine patterns in plasma and sputum in hospitalized patients with CAP at the time of hospital admission. We found that approximately half of the patients were in each inflammatory response group across analysis of sputum cytokines, plasma cytokines, and the plasma/sputum cytokine ratio. Several patients were retained in the same group in all 3 analyses. Since this was a pilot study, larger studies may identify more than the two patterns identified in our study. The only clinically relevant difference identified in the patient characteristics comparisons between high vs low inflammatory phenotypes was the need for direct ICU admission when comparing both sputum and plasma cytokines. In both comparisons, patients with lower inflammatory phenotypes necessitated more intensive care on admission compared to those with higher inflammatory phenotypes. With our small sample size it is unclear as to if these differences are real or due to chance however this may be an interesting variable to evaluate in future larger studies. For sputum cytokines, it is possible that patients with a lower inflammatory response on admission have a poor local immune response and may end with a more severe disease leading to ICU admission.

A timeline of the inflammatory response in patients with pneumonia can be generalized as follows (**Figure 2**): At time zero, during the initial invasion of organisms into the alveolar space, there is

an interaction of the invading pathogen with cells of the innate immune system. The identification of the pathogen is made by receptors called pattern recognition receptors (PRR). Streptococcus pneumoniae, as well as other extracellular pathogens may be recognized by a family of membrane-bound PRPs called Toll-like receptors. Influenza and other intracellular pathogens may be recognized by a family of cytoplasmic PRPs called NOD-like receptors.

During the initial 24 hours after infection, activation of theses receptors will trigger synthesis and secretion of cytokines. Since different pathogens are recognized by different receptors with different signaling mechanisms, it is predictable that the initial inflammatory response may be pathogen specific. On the other hand, the inflammatory response will also be host specific, as the initial cytokine response will be different in patients with different underlying medical conditions, who have chronic inflammation, or who are taking medications that may modulate the inflammatory response. Due to the multiple pathogen and host factors that may influence the inflammatory response, the initial cellular response and cytokine pattern is likely to be pathogen and host specific.

On days two and three post infection, a well-developed lung inflammatory response should be present. The initially specific inflammatory response may begin to evolve into non-specific inflammatory pathways. At this time, the local lung inflammatory response may spill over into the circulatory system, generating a systemic inflammatory response.

Patients with pneumonia are hospitalized with an average of 3 to 5 days of signs and symptoms. By this time, we hypothesized that patients present with two patterns of inflammatory response, one associated with repair, and another associated with injury, as depicted in **Figure 2**. We further hypothesized that these patterns of response can be identified by performing a cluster analysis of cytokines at the time of hospital admission. In the current pilot study, we were able to characterize patterns of local (sputum) and systemic (plasma) inflammatory response in hospitalized patients with pneumonia.

We speculate that patients with an exaggerated inflammatory pattern only in the sputum may not be ideal candidates for clinical trials of immunomodulatory therapies, since a local immune response is an appropriate physiologic response to pneumonia. Reducing a non-exaggerated immune response may not be therapeutic, could bias a trial's results to the null, and could even lead to poor outcomes. However, patients with an exaggerated inflammatory response in the circulation may be excellent candidates for these trials due to their systemic cytokine storm. As depicted in **Figure 2**, 8 of the 13 CAP patients in our study appeared to have an exaggerated inflammatory response in plasma. These patients would be appropriate candidates for immunomodulation.

Since this was a pilot study, we enrolled a limited number of patients. Evaluating only these 13 patients limits the generalizability. Furthermore, this small sample size allows for potentially biased cluster analyses. Another limitation of our study is that the line separating our definition of the exaggerated inflammatory re-
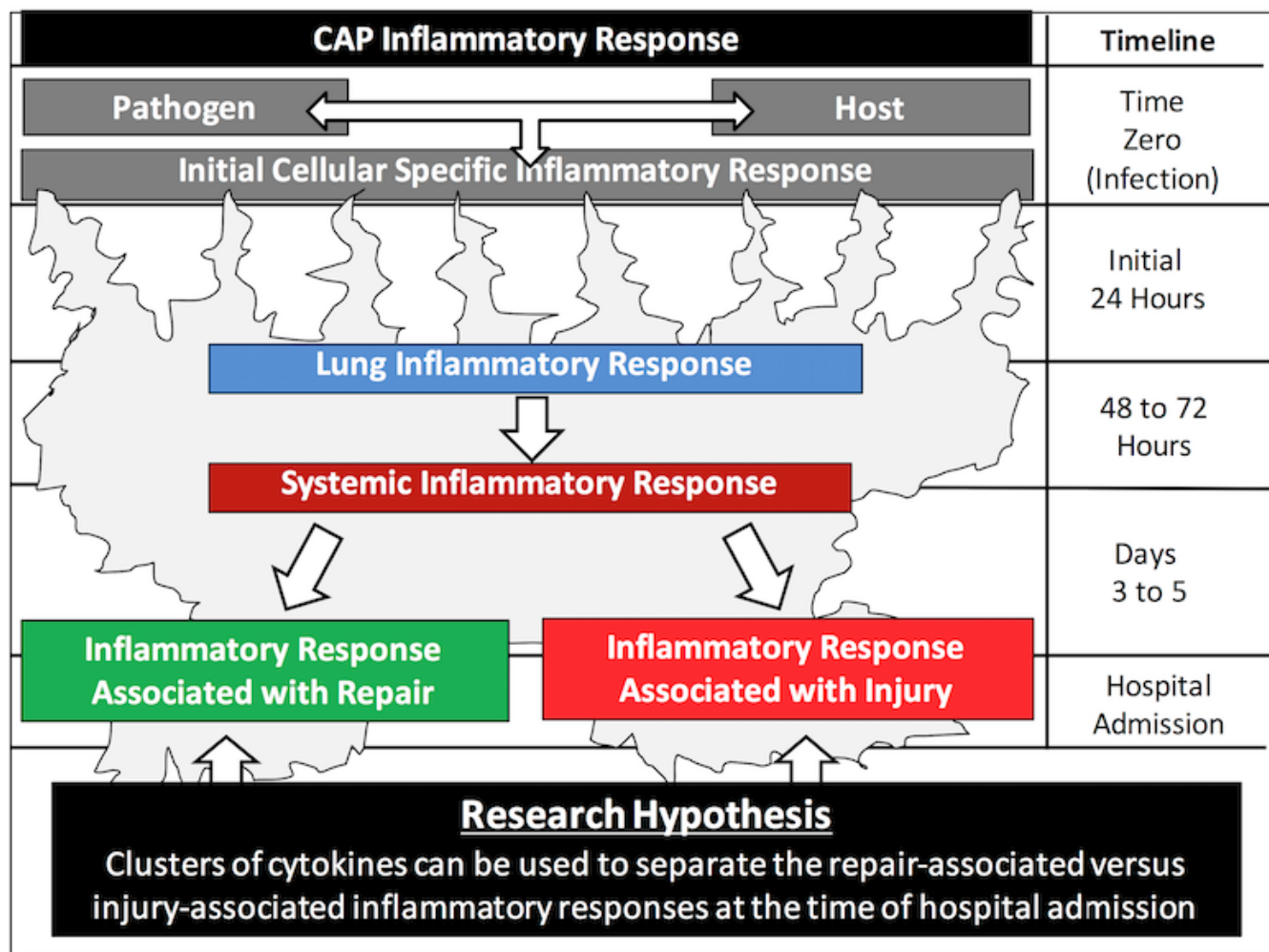
Fig. 2 A timeline of the inflammatory response in patients with pneumonia

sponse was partially based on visual inspection of the heatmap. This may lead to misclassification of some patients due to the partially subjective nature of this classification. Not all aspects of inflammation and anti-inflammation were evaluated in this study. Other makers may be important for determining an exaggerated inflammatory response to infection. Another limitation is that the bacterial burden was not measured. This can influence inflammatory response and bias the clustering results. One possible strength of our study is the ability of the cluster analysis to normalize cytokine values without dependence on a single cytokine value that may be pro- or anti-inflammatory on its own.

These interesting results suggest a prospective study with a larger number of patients is warranted, with the goal to characterize patterns of inflammatory response in blood and sputum in patients with CAP at the time of hospital admission. In this study, it will be important to evaluate variables that may confound the inflammatory response such as etiology of CAP, the use of steroids, and the use of macrolide antibiotics. Furthermore, if cytokine values were to be determined immediately upon admission each value could be automatically uploaded to and analyzed by a cloud-based application using supervised machine learning algo-

rithms. Results of the analysis could be reported back to a mobile device in real-time as a yes/no decision rule. This would allow for rapid determination of the level of inflammation to define candidacy into clinical trials for immunomodulatory therapies.

In conclusion, we were able to define patterns of inflammatory response using cytokine data in this pilot study. Our results suggest that airway and systemic inflammation in CAP is heterogeneous, providing proof of principle that stratification based on the inflammatory patterns may be possible for future trials of immunomodulation.

research. TLW, RRK, WAM, RFB, and JAR conducted the data analysis. All authors were involved in data interpretation. TLW and JAR wrote the first draft of the manuscript. All authors critically reviewed the manuscript for important intellectual content. All authors agree with the manuscript results and conclusions.

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