Is Genomic Medicine Finally Coming of Age for the Diagnosis of Pneumonia?

The rapid diagnosis of community-acquired pneumonia (CAP) today relies on a compatible history and physical examination combined with a routine chest radiograph and a Gram stain. The Danish microbiologist Hans Christian Gram (1838–1938) first introduced his famous stain to clinical medicine in 1884 (1). He initially proposed its value in identifying bacterial pathogens from white blood cells and sputum debris. He and other microbiologists quickly recognized that the Gram stain also conveniently divided the microbial world essentially into two halves, with bacteria containing lipopolysaccharide in their cell walls staining pink (gram-negative bacteria) and those without lipopolysaccharide staining dark blue (gram-positive bacteria). German physicist and Nobel Prize laureate Wilhem Conrad Röntgen (1845–1923) first reported the discovery and potential clinical applications of what he called X-rays in 1896 (2). Surprisingly, the initial diagnostic approach to identifying bacterial pneumonia has not improved appreciably in the 21st century from what it was back in the 19th century.

The relative absence of progress in improving the rapid and accurate diagnosis of community-acquired pneumonia (CAP) is not for lack of trying. A myriad of diagnostic biomarkers have been proposed to assist in the diagnosis of CAP and include inflammatory plasma protein markers, antigen detection systems, and rapid pathogen detection methods using nucleic-based technologies (3–7). Some of these biomarkers and selected combinations of biomarkers may have clinical merit, but their overall effect in the standard diagnostic approach to CAP remains limited.

Accurate and timely diagnosis of lower respiratory infection is a major unmet medical need. Early institution of appropriate antibiotics for severe pneumonia can be lifesaving (8). However, the profligate use of empiric, broad-spectrum antibiotics for suspected severe CAP (SCAP, which is operationally defined as CAP of sufficient severity to warrant intensive care unit admission) likely contributes to antimicrobial selection pressures in the intensive care unit environment that promotes the spread of multidrug-resistant bacterial pathogens (9, 10).

In this issue of the *Journal*, Scicluna and colleagues (pp. 826–835) propose to improve this situation with a new molecular diagnostic biomarker for the rapid diagnosis of SCAP (11). These investigators began by using a microarray analysis of the transcriptome from circulating white blood cells. They compared patients with SCAP with severely ill patients with respiratory symptoms suggestive of SCAP who were later determined not to have pneumonia. They interrogated their predictive gene arrays from a derivation cohort followed by a separate validation cohort. A total of 171 patients with SCAP and 63 critically ill non-SCAP patients were used as the comparator.

They defined a gene signature profile of 78 genes that distinguished patients with SCAP from non-SCAP patients from the more than 9,000 differentially expressed genes compared from healthy subjects. Of the initial set of 78 selected gene transcripts, a ratio of two gene transcripts, *FAIM3*:*PLAC8*, proved to be the most robust discriminator between patients with SCAP and non-SCAP patients. The receiver operating characteristic of the area under the curve of this ratio was a very respectable 0.845, with a positive and negative predictive value of 83% and 81%. The posttest probability of a patient having SCAP if the test was positive was 83%.

The authors should be congratulated for performing such a careful study and using the optimal comparator for SCAP, which is acutely ill intensive care unit patients with respiratory complaints but who turn out not to have SCAP. Moreover, they chose a rather simple molecular biomarker ratio consisting of only two transcription targets from whole blood, facilitating the later development of a point-of-care, PCR-based diagnostic test (12). The two genes chosen from this investigation were both negative regulators of apoptosis: *FAIM3* (fas apoptotic inhibitory molecule 3), and *PLAC8* (placenta specific 8). The *FAIM3*:*PLAC8* ratio outperformed existing plasma protein biomarkers IL-6, IL-8, and procalcitonin for the diagnosis of severe CAP. The results from the discovery cohort (n = 134) were remarkably similar to the findings in the validation cohort (n = 100), which is encouraging indeed.

Despite the numerous favorable attributes of this clinical and genomic study, caution must be exercised in analyzing the potential diagnostic and therapeutic value of this genomic biomarker. The total population of patients in this study is still relatively small, and the positive likelihood ratio (true-positive/false-positive) of the diagnostic test was 1.62, which is modest at best, despite the high pretest probability that SCAP is present in the study population. A larger sample size would help ensure precision and accuracy, as would the inclusion of patients with varied genetic backgrounds from other regions of the world.

Another issue is the "gold standard" determination of the final diagnosis. The degree of agreement between the clinician judges in defining SCAP from other respiratory diseases in these acutely ill patients, according to a retrospective review of all the existing clinical and microbiologic data, was good but not perfect. The kappa value was 0.85. Even with all the information available, our ability to consistently agree on a final diagnosis of SCAP is imperfect. Misclassified patients could adversely affect the calculated diagnostic accuracy of the transcript biomarker test.

We also have to acknowledge that previous attempts to develop a reproducible, simple, gene array assay to distinguish systemic inflammation from invasive infection in other indications such as trauma, sepsis, and ventilator-associated pneumonia has met with limited success and remains an active area of research (13–15). Finally, what actions would the clinician do differently if this genomic biomarker were made available? Certainly, the information would be useful in providing a risk assessment about

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possible SCAP. However, would a decision be made to withhold antibiotics in critically ill patients with suspected SCAP if the test were negative? As the authors point out (11), the positive predictive value of the assay (probability that SCAP is present when the assay is positive) is currently 83%, whereas the negative predictive value (likelihood that SCAP is absent when the test is negative) is 81%. This indicates that you cannot exclude the possibility of SCAP with the assay. It is unlikely that confidence in a negative test is such that antibiotics would be discontinued, placing critically ill patients at risk for untreated SCAP.

Will this genomic diagnostic test finally reach a threshold on which we can base our therapeutic decisions with greater certainty than relying on the usual clinical parameters and single or multiple protein biomarkers? Not at present, but this work is now moving in the right direction, and perhaps further improvements in this or similar assays will improve the test performance and diagnostic accuracy. Perhaps we do not have to look too far in the future to a time when intensive care units will have these assays available, and they will be of sufficient reliability to have confidence in making therapeutic choices guided by real-time genomic testing. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Steven Opal, M.D.
Alpert Medical School of Brown University
Providence, Rhode Island
and
Infectious Disease Division
Memorial Hospital of Rhode Island
Pawtucket, Rhode Island

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The Evidence for Long-Term Benefits of Restoration of CFTR Function Continues to Grow

In 1989, it was first reported that mutations in the *CFTR* gene on chromosome 7 result in dysfunction of the CFTR protein and cause the multisystem disorder we know as cystic fibrosis (CF) (1). Although that initial discovery offered hope of treating the underlying cause of CF by restoring CFTR protein function, it was not until 2012, when Ramsey and coworkers reported the effect of the CFTR potentiator ivacaftor in a subset of patients with CF and the G551D mutation, that this hope was realized (2). In that study, restoring CFTR function with ivacaftor resulted in significant improvement in lung function, reduction in pulmonary exacerbations, and improvement in body mass during a 48-week study period. Subsequent studies demonstrated additional benefits of CFTR function restoration with ivacaftor in G551D patients, including reduction in hospitalizations, reduction in the prevalence of *Pseudomonas aeruginosa* in respiratory cultures, and improved growth (3, 4). But the rapid implementation of ivacaftor as standard of care for all patients with CF with a G551D mutation (5), and the resulting lack of an untreated G551D group for comparison, made it difficult to answer one essential question: Does CFTR function restoration by ivacaftor result in a benefit beyond just the initial improvement in lung function and actually reduce the long-term rate of decline in lung function that is characteristic of CF? In other words, is ivacaftor truly "disease-modifying"?

ORIGINAL ARTICLE

A Molecular Biomarker to Diagnose Community-acquired Pneumonia on Intensive Care Unit Admission

Brendon P. Scicluna[1], Peter M. C. Klein Klouwenberg[2,3,4]*, Lonneke A. van Vught[1]*, Maryse A. Wiewel[1], David S. Y. Ong[2,3,4], Aeilko H. Zwinderman[5], Marek Franitza[6,7], Mohammad R. Toliat[6], Peter Nürnberg[6,7,8], Arie J. Hoogendijk[1], Janneke Horn[9], Olaf L. Cremer[2], Marcus J. Schultz[9], Marc J. Bonten[3,4], and Tom van der Poll[1,10]

[1]Center for Experimental Molecular Medicine and Center for Infection and Immunity Amsterdam, [5]Clinical Epidemiology Biostatistics and Bioinformatics, [9]Department of Intensive Care Medicine, and [10]Division of Infectious Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; [2]Department of Intensive Care Medicine, [3]Department of Medical Microbiology, and [4]Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, the Netherlands; and [6]Cologne Center for Genomics, [7]Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases, and [8]Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany

Abstract

Rationale: Community-acquired pneumonia (CAP) accounts for a major proportion of intensive care unit (ICU) admissions for respiratory failure and sepsis. Diagnostic uncertainty complicates case management, which may delay appropriate cause-specific treatment.

Objectives: To characterize the blood genomic response in patients with suspected CAP and identify a candidate biomarker for the rapid diagnosis of CAP on ICU admission.

Methods: The study comprised two cohorts of consecutively enrolled patients treated for suspected CAP on ICU admission. Patients were designated CAP (cases) and no-CAP patients (control subjects) by *post hoc* assessment. The first (discovery) cohort (101 CAP and 33 no-CAP patients) was enrolled between January 2011 and July 2012; the second (validation) cohort (70 CAP and 30 no-CAP patients) between July 2012 and June 2013. Blood was collected within 24 hours of ICU admission.

Measurements and Main Results: Blood microarray analysis of CAP and no-CAP patients revealed shared and distinct gene expression patterns. A 78-gene signature was defined for CAP, from which a *FAIM3*:*PLAC8* gene expression ratio was derived with area under curve of 0.845 (95% confidence interval, 0.764–0.917) and positive and negative predictive values of 83% and 81%, respectively. Robustness of the *FAIM3*:*PLAC8* ratio was ascertained by quantitative polymerase chain reaction in the validation cohort. The *FAIM3*:*PLAC8* ratio outperformed plasma procalcitonin and IL-8 and IL-6 in discriminating between CAP and no-CAP patients.

Conclusions: CAP and no-CAP patients presented shared and distinct blood genomic responses. We propose the *FAIM3*:*PLAC8* ratio as a candidate biomarker to assist in the rapid diagnosis of CAP on ICU admission.

Clinical trial registered with www.clinicaltrials.gov (NCT 01905033).

Keywords: sepsis; pneumonia; blood; biomarker; microarray

Community-acquired pneumonia (CAP) is associated with significant mortality worldwide (1) and accounts for up to 44% of severe sepsis (2, 3). Patients requiring intensive care unit (ICU) admission represent 10–15% of CAP cases (4, 5), in whom mortality can reach 20–25% in those needing vasopressor support (6). Although CAP is clinically well-defined, the occurrence of noninfectious causes of respiratory distress complicates the diagnosis, often leading to a delay in appropriate therapeutic management of patients (7). Delays in antimicrobial treatment of critically ill infectious patients

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*These authors contributed equally.

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Correspondence and requests for reprints should be addressed to Brendon P. Scicluna, Ph.D., Academic Medical Center, Center of Experimental and Molecular Medicine, Meibergdreef 9, Room G2-130, 1105AZ Amsterdam, the Netherlands. E-mail: b.scicluna@amc.uva.nl

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At a Glance Commentary

Scientific Knowledge on the Subject: Rapid and adequate identification of severe community-acquired pneumonia (CAP) is important for timely initiation of cause-specific therapy. Protein biomarkers provide insufficient diagnostic accuracy for use in clinical practice.

What This Study Adds to the Field: This investigation comprised two independent cohorts of patients treated for suspected CAP on admission to the intensive care unit, which for the purpose of this study were each divided into CAP (cases) and no-CAP (control subjects) patients by *post hoc* diagnostic stratification. Although we show a tremendous blood leukocyte genomic response in CAP and similarly in no-CAP patients, we describe the discovery and validation of a combinatorial quantitative host blood genomic biomarker test that can assist in the rapid identification of severe CAP.

have been associated with prolonged length of stay in ICUs and heightened the risk of mortality by 8.5% within 4–6 hours of ICU admission (8, 9). Thus, rapid and adequate identification of CAP on admission to the ICU is of outstanding importance.

Considerable research has been conducted to accurately distinguish patients with sepsis from those with noninfectious causes of disease, predominantly focused on plasma proteins procalcitonin, soluble triggering receptor expressed on myeloid cells 1, and IL-8 and -6 (10–12). Although some protein biomarkers may be of value in identifying patients with bacterial CAP, their clinical value in the setting of severe CAP requiring ICU admission is limited (13, 14). Technological innovations have positioned systems biology at the forefront of biomarker discovery (15, 16). Analysis of the whole-blood leukocyte transcriptome enables the assessment of thousands of molecular signals beyond simply measuring several proteins in plasma, which for use as biomarkers is important because combinations of biomarkers likely provide more diagnostic accuracy than the measurement of single ones or a few (17–19).

Evidence suggests that genome-wide transcriptional profiling of blood leukocytes can assist in differentiating between infection and noninfectious causes of severe disease (20, 21). Of importance, RNA biomarkers have the potential advantage that they can be measured reliably in rapid quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)-based point-of-care tests (15, 22). Here, through the analysis of whole-blood leukocyte transcriptional profiles we aimed to characterize the systemic host response in patients with severe CAP and to identify and validate a candidate diagnostic molecular signature for the differential diagnosis of CAP and noninfectious ICU patients treated for suspected CAP.

Methods

Study Design and Patient Selection

The study was performed within the context of the Molecular Diagnosis and Risk Stratification of Sepsis project in two tertiary referral centers in the Netherlands (Academic Medical Center, Amsterdam, and University Medical Center Utrecht, Utrecht) (23, 24). The Medical Ethics committees of both participating centers approved an opt-out consent method (IRB no. 10–056C). The current study comprised two cohorts of consecutively enrolled patients admitted to the ICU with suspected CAP for which the attending physician started antibiotic therapy. CAP diagnosis was based on International Sepsis Forum Consensus Conference definition (25), and described in detail previously (*see* Table E1 in the online supplement) (24). Dedicated observers classified the plausibility of CAP as "definite," "probable," "possible," or "none" based on a *post hoc* review of all available clinical, radiologic, and microbiologic evidence as described (24). Interobserver agreement for the diagnosis and likelihood of CAP was good (kappa, 0.85) (24). Patients with a *post hoc* CAP likelihood of "definite" or "probable" were used as CAP (cases); patients with a *post hoc* CAP likelihood of "none" were used as no-CAP (control subjects). No-CAP patients did not have an infection from a different source either.

The first (discovery) cohort was enrolled between January 2011 and July 2012, and included 101 CAP and 33 no-CAP patients. The second (validation) cohort was enrolled between July 2012 and June 2013, and included 70 CAP and 30 no-CAP patients. Exclusion criteria are presented in the online supplement. Severity was assessed by the Acute Physiology and Chronic Health Evaluation (APACHE) IV score (26). Shock was defined as hypotension requiring noradrenaline (>0.1 μg/kg/min) during at least 50% of the day. Blood was collected in PAXgene tubes (Becton-Dickinson, Breda, the Netherlands) and ethylenediaminetetraacetic acid vacutainer tubes within 24 hours of ICU admission. PAXgene blood samples were also obtained from 42 healthy control subjects (median age, 35 [interquartile range, 30–63] yr; 57% male) after providing written informed consent.

Microarrays and qRT-PCR

PAXgene blood RNA isolation, microarray, and qRT-PCR analyses are described in the online supplement. For microarrays, RNA was hybridized to the Human Genome U219 96-array plate and scanned using the GeneTitan instrument (Affymetrix, High Wycombe, UK).

Immunoassays

Plasma (ethylenediaminetetraacetic acid) was used for procalcitonin measurements by Kryptor (Thermo Fisher, Brahms GmbH, Hennigsdorf, Germany), and IL-8 and -6 measurements by cytometric bead arrays (BD Biosciences, Breda, the Netherlands).

Statistical Analysis

Statistical analysis was performed in the R statistical environment (version 3.2.0, R Foundation for Statistical Computing, Vienna, Austria). Categorical variables were evaluated by Fisher exact tests, whereas continuous variables were analyzed by Wilcoxon rank sum test. Receiver operating characteristic curve including area under the curve (AUC) analysis, confidence interval (CI), accuracy, and positive and negative predictive values were analyzed using the *pROC* package (27). The 95% CIs for the calculated AUCs were estimated by bootstrap. Significance was demarcated at P less than 0.05. Threshold-dependent positive and negative likelihood ratios (LR+ and LR−, respectively) and Bayesian post-test probabilities were calculated in

R (version 3.2.0). We present data in the form of Venn-Euler plots, volcano plots, heatmap plots, hierarchical clustering, 3D principal component, and dot plots.

Results

Patient Characteristics

CAP and no-CAP patients enrolled in the discovery cohort (for blood leukocyte analyses) are described in Table 1. CAP and no-CAP patients were largely similar in demographics, comorbidities, treatment, and outcome. The presence of shock, the need for mechanical ventilation, and mortality did not differ between groups. Notably, antibiotic therapy was similar in CAP and no-CAP patients with the exception of more ciprofloxacin treatments in the former group, illustrating the clinical suspicion for CAP in all patients. The most common final diagnoses in no-CAP patients were suspected aspiration, exacerbation of chronic obstructive pulmonary disease or asthma, and congestive heart failure (*see* Table E3).

Shared and Distinct Leukocyte Genomic Signatures Define CAP and No-CAP Patients

Global gene expression profiles of whole-blood leukocytes collected within 24 hours after ICU admission from CAP and no-CAP patients were compared with those of healthy individuals. Differential gene expression analysis showed CAP and

Table 1. Characteristics of the CAP Patients (Cases) and No-CAP Patients (Control Subjects) in Discovery and Validation Cohorts

Parameter	Discovery Cohort			Validation Cohort		
	No-CAP Patients (*n* = 33)	CAP Patients (*n* = 101)	*P* Value	No-CAP Patients (*n* = 30)	CAP Patients (*n* = 70)	*P* Value
Demographics						
Age, Mdn (IQR), yr	59 (48–67)	64 (52–73)	0.058*	61 (49–74)	63 (19–73)	0.58*
Sex, male, %	67	57	0.42†	53	57	0.83†
Core temperature, Mdn (IQR), °C	37.8 (37.1–38)	37.7 (37.1–38.3)	0.7*	37.5 (36.6–37.7)	38 (37.1–38.5)	0.037*
Comorbidities						
Diabetes mellitus, %	18	23	0.64†	27	24	0.81†
COPD, %	15	26	0.24†	30	24	0.74†
Hypertension, %	36	31	0.67†	37	30	0.64†
Hematologic malignancy, %	3	8	0.45†	4	6	1†
Severity indices						
APACHE IV score, Mdn (IQR)	74 (49–112)	81 (64–97)	0.54*	61 (52–84)	78 (60–100)	0.012*
Shock, %	15	29	0.17†	0	21	0.005†
Causal pathogens						
Streptococcus pneumoniae, %	—	23	na	—	27	na
Staphylococcus aureus, %	—	13	na	—	7	na
Haemophilus influenzae, %	—	11	na	—	4	na
Pseudomonas aeruginosa, %	—	5	na	—	7	na
Other gram-positive bacteria, %	—	7	na	—	3	na
Other gram-negative bacteria, %	—	9	na	—	7	na
Pneumocystis jirovecii, %	—	4	na	—	0	na
Aspergillus fumigatus, %	—	1	na	—	3	na
Unknown, %	—	33	na	—	26	na
Treatment						
Mechanical ventilation, %	76	69	0.52†	73	70	0.81†
Empirical antibiotic treatment						
Cefotaxim, %	39	42	1†	67	37	0.001†
Erytromycin, %	18	38	0.054†	33	30	0.81†
Ceftriaxon, %	15	25	0.34†	20	37	0.11†
Ciprofloxacin, %	3	21	0.015†	7	41	0.0004†
Amoxicillin/clavulanic acid, %	9	9	1†	10	6	0.43†
Oseltamivir, %	9	25	0.08†	33	54	0.08†
Other, %	24	34	0.65†	13	30	0.01†
Outcomes						
ICU mortality, %	21	15	0.42†	7	22	0.09†
Hospital mortality, %	24	24	1†	17	30	0.22†
30-d mortality, %	21	23	0.91‡	20	27	0.47‡
ICU LoS, Mdn (IQR), d	3 (1–4)	5 (2–11)	0.001*	1 (1–3)	5 (2–12)	0.001*
Hospital LoS, Mdn (IQR), d	11 (4–23)	15 (9–23)	0.055*	7 (5–12)	13 (7–28)	0.024*

Definition of abbreviations: APACHE IV = Acute Physiology and Chronic Health Evaluation IV score (26); CAP = community-acquired pneumonia; COPD = chronic obstructive pulmonary disease; ICU = intensive care unit; IQR = interquartile range; LoS = length of stay; Mdn = median; na = not applicable. Significance was demarcated at $P < 0.05$.

*Wilcoxon rank sum test probability.

†Fisher exact test probability.

‡Chi-square test probability.

no-CAP patient populations each presented marked changes in their blood leukocyte response as compared with healthy subjects. A total of 9,274 genes were significantly altered (using a Benjamini-Hochberg [BH] adjusted $P < 0.05$) in CAP (Figure 1A). Of these genes, 48% were overexpressed, whereas 52% were underexpressed. These dramatic changes in blood leukocyte gene expression were also evident in no-CAP patients with 5,772 altered genes, of which 39% were overexpressed and 61% underexpressed genes. Remarkably, 5,391 genes were similarly altered in expression in CAP and no-CAP patients when compared with healthy subjects (Figure 1A).

Pathway analysis revealed those common overexpressed and underexpressed genes were significantly associated (BH adjusted probability, <0.05) with canonical signaling pathways (*see* Figure E1). Common overexpressed pathways included typical proinflammatory, antiinflammatory, mitochondrial dysfunction pathways, and molecular mechanisms involved in cancer (*see* Figure E1A). Underexpressed genes associated with hereditary breast cancer signaling pathways, transfer RNA charging (mRNA translation pathway), protein ubiquitination (protein elimination), and metabolic pathways (*see* Figure E1B). These findings suggest that blood leukocyte genomic responses in CAP and no-CAP patients were predominantly similar, characterized by heightened expression of proinflammatory and antiinflammatory pathways paralleled by a decrease in protein production/elimination and cellular energy-generating pathways. The enrichment for cancer-related pathways also suggests the common host blood genomic response may not be confined solely to critical illness but also to other life-threatening diseases, such as cancer (28).

CAP and no-CAP patients also exhibited uniquely altered genes when respectively compared with healthy subjects, where CAP patients presented 3,883 uniquely altered gene expression profiles (Figure 1A). Overexpressed genes associated to canonical signaling pathways that included clathrin-mediated endocytosis and caveolar-mediated endocytosis (Figure 1B), whereas underexpressed genes associated to pathways that included Cdc42 signaling, IL-3, and apoptosis signaling (Figure 1B). A comparative analysis of CAP and no-CAP patients (moderated *t* statistics) identified 2,459 significantly altered genes (BH adjusted, $P < 0.05$) (Figure 1C). Underexpressed genes in CAP associated to pathways that included EIF2 signaling (protein translation), T-cell receptor signaling, and mTOR signaling (Figure 1D). The differential gene expression profiles between CAP and no-CAP patients (2,459 genes) (Figure 1C) were subsequently used as the foundation for derivation of a candidate CAP signature and biomarker.

Identification of Molecular Biomarkers Discriminating CAP from No-CAP Patients

The methodologic steps used in generating an ICU gene expression signature for CAP and identifying a candidate diagnostic biomarker are outlined in Figure E2. Using a nearest shrunken centroid fit and 10-fold cross-validation (*see* Figure E3A), a 78-gene expression signature was delineated (cross-validation error rate, 14.9%) (*see* Figure E3B) for CAP and no-CAP patients discrimination (Figure 2A). Decomposing the 78-gene signature into 12 principal components (*see* Figure E3C) and considering the first three major principal components revealed a cumulative explainable variance of 70.1% (principal component 1, 50.5%; principal component 2, 11.6%; principal component 3, 5.6%) (Figure 2B). This analysis confirmed discrimination of CAP patients from no-CAP patients and revealed molecular heterogeneity in the cohort, which was also evident after unsupervised hierarchical clustering (*see* Figure E3D).

Considering our 78-gene CAP signature we assessed all ratios of genes, ranked by AUCs and Wilcoxon rank sum test BH-adjusted significance for the discrimination of CAP and no-CAP patients. The top ranked identified ratio was *FAIM3*:*PLAC8* with threshold-independent receiver operating characteristic AUC of 0.845 (95% CI, 0.764–0.917), which was also higher than the 78-gene signature AUC of 0.749. A numerical threshold for the *FAIM3*:*PLAC8* gene ratio test was defined at 0.757 (Figure 2C), which favored high sensitivity (97%) but at the expense of specificity (40%). By favoring a high sensitivity we sought to address the potentially serious consequences of false-negative predictions (CAP patient classified as no-CAP). Thus, at the predefined threshold our *FAIM3*:*PLAC8* gene ratio test yielded 83.1% positive predictive value (95% CI, 79.4–87.4%), 81.3% negative predictive value (95% CI, 62.5–100%), and 82.8% accuracy (95% CI, 77.6–87.3%).

To explore the association between our *FAIM3*:*PLAC8* gene expression ratio and white blood cell counts and differentials, we first evaluated the differences between CAP and no-CAP patients. Most notably, only lymphocyte counts were significantly different, with higher counts in no-CAP patients (*see* Figure E4A). Second, Spearman correlation analyses showed *FAIM3*:*PLAC8* ratios correlated to lymphocyte (rho = 0.4; $P = 6.9 \times 10^{-5}$) and eosinophil counts (rho = 0.34; $P = 0.0007$) (*see* Figure E4B).

The Leukocyte Genomic Response at ICU Admission Does Not Discriminate between CAP Survivors and Nonsurvivors

Univariate analysis demonstrated that blood genomic patterns marginally differed between CAP survivors and nonsurvivors. Only three genes were significantly different between ICU survivors and nonsurvivors (BH adjusted, $P < 0.05$); no genes were statistically different between survivors and nonsurvivors at Day 30 (*see* Figure E5). These results indicate that a molecular signature is unlikely to assist in predicting the risk of dying in CAP patients.

Clinical Utility and Comparison of *FAIM3*:*PLAC8* Gene Expression Biomarker with Protein Biomarkers

Several protein biomarkers have been evaluated for their capacity to discriminate between sepsis and noninfectious causes of critical illness, including procalcitonin and IL-6 and -8 (10–12). Admission plasma procalcitonin and IL-6 and -8 concentrations were significantly different between CAP and no-CAP patients (Figure 3A). AUC analysis showed these protein biomarkers were not suitable for the discrimination of CAP and no-CAP patients, either in isolation or in combination with our *FAIM3*:*PLAC8* gene expression ratio (Figure 3B). LR+, LR−, and Bayesian post-test probabilities of the FAIM3:PLAC8 ratio, procalcitonin, and IL-6 and -8 are shown in Table E4. These results indicated that the *FAIM3*:*PLAC8* ratio performed better in diagnosing CAP than plasma proteins, with LR+ 1.62, LR− 0.075, and post-test

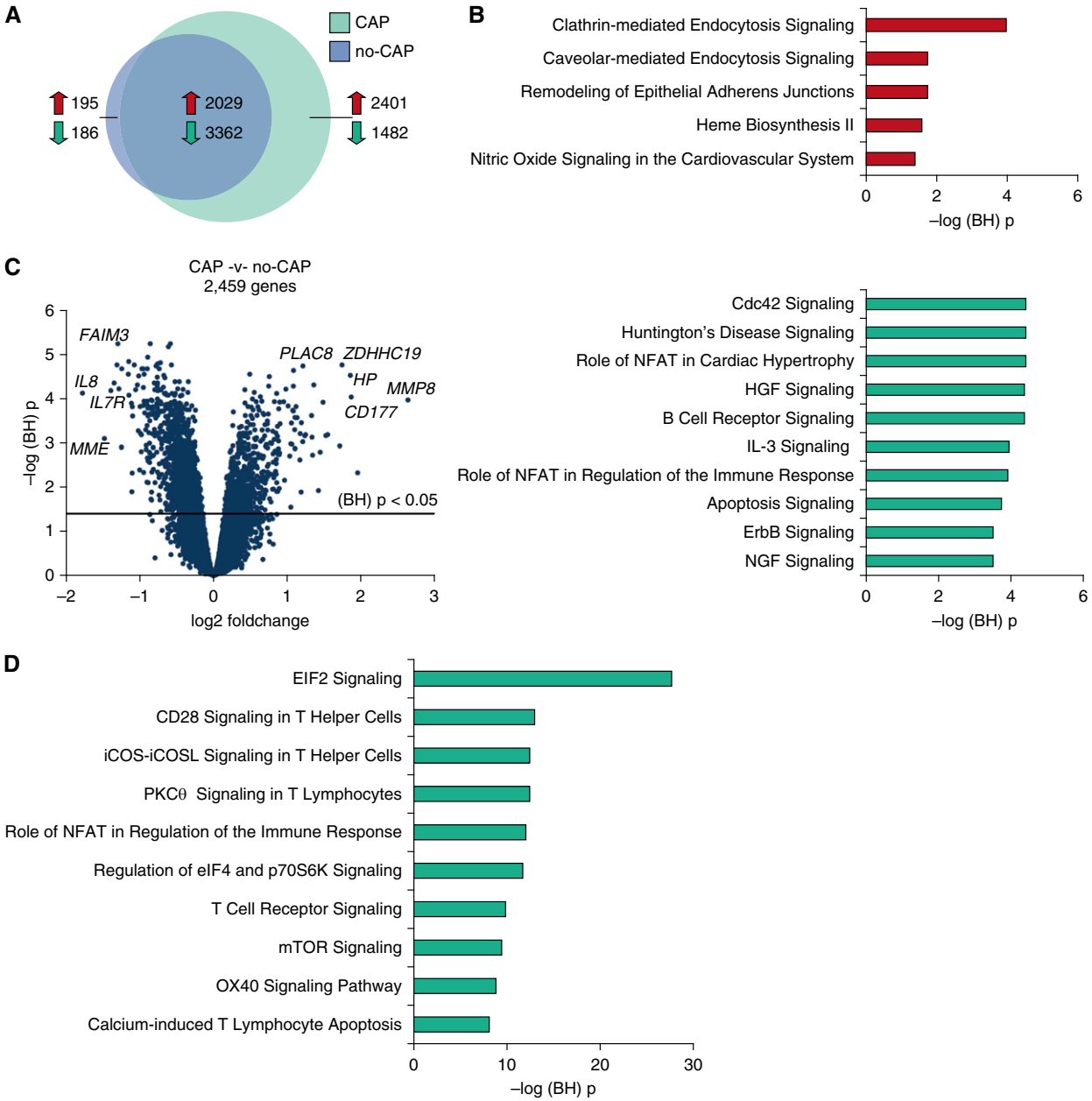

Figure 1. Blood genomic responses in consecutively enrolled patients treated for suspected community-acquired pneumonia (CAP) on intensive care unit admission. (*A*) Venn-Euler representation of differentially expressed genes in CAP and no-CAP (noninfectious control subjects) patients versus healthy subjects (Benjamini-Hochberg [BH]-adjusted, $P < 0.05$). *Red arrows* denote overexpressed genes; *green arrows* denote underexpressed genes. (*B*) Ingenuity pathway analysis of overexpressed (n = 2,401, *red*) and underexpressed (n = 1,482, *green*) genes unique to CAP patient genomic responses. –log (BH)p = negative $\log_{10}$-transformed BH-adjusted Fisher exact *P* value. (*C*) Volcano plot representation (integrating log2 fold changes and *P* values) of the CAP and no-CAP patient comparison. A total of 2,459 significantly altered genes (BH-adjusted, $P < 0.05$) were identified. (*D*) Ingenuity pathway analysis of the 2,459 significantly altered genes revealed significant associations (BH-adjusted, $P < 0.05$) of underexpressed genes with canonical signaling pathways. No statistically significant pathway association was identified for overexpressed genes.

probabilities of being CAP positive or negative at 83% and 19%, respectively (*see* Table E4). These findings showed our gene expression *FAIM3*:*PLAC8* biomarker was superior to plasma protein biomarker abundances in discriminating CAP and no-CAP patients treated for suspected CAP.

Validation of the CAP Molecular Biomarker in an Independent ICU Cohort

To ascertain robustness of the *FAIM3*:*PLAC8* ratio as a candidate biomarker we tested it in an independent ICU cohort by qRT-PCR. The patient characteristics of the validation cohort are shown in Table 1. As with the discovery cohort, in the validation cohort CAP and no-CAP patients were similar in demographics, comorbidities, need for mechanical ventilation, and mortality. CAP patients had significantly higher APACHE IV

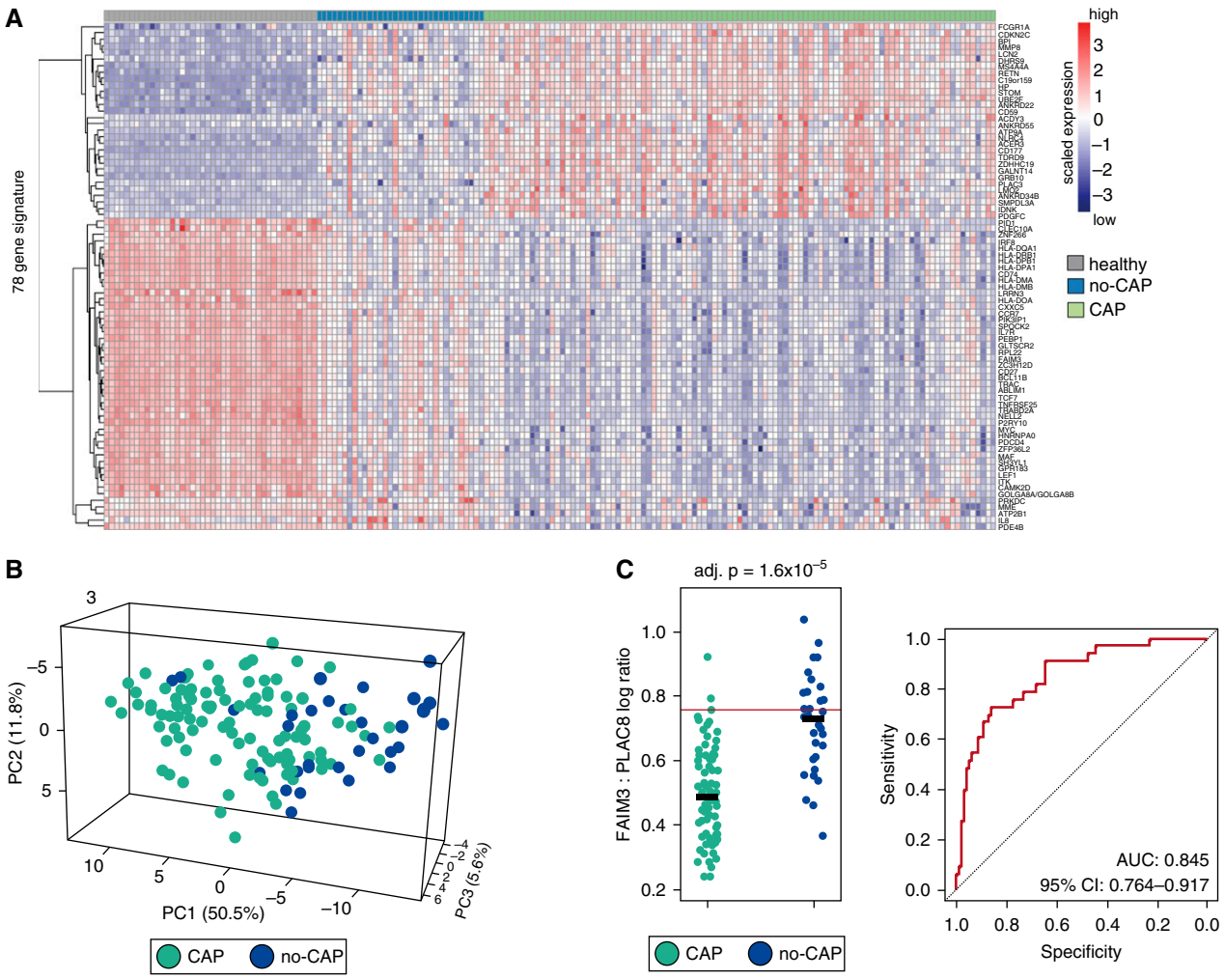

Figure 2. Community-acquired pneumonia (CAP) genomic signature and gene expression classifier. (*A*) Supervised heatmap plot of the 78-gene expression CAP signature defined by shrinked centroid and 10-fold cross-validation classification of 2,459 differentially expressed genes (Benjamini-Hochberg adjusted, $P < 0.05$). *Columns* represent healthy subject, CAP (cases), and no-CAP (control subjects) patient samples. *Rows* represent scaled gene expression values. *Red* = overexpressed; *blue* = underexpressed. (*B*) Three-dimensional principal component analysis plot depicting the first three major components and revealing a cumulative explainable variance of 70.1% for the 78-gene expression CAP classifier. PC = principal component. (*C*) Dot plot and receiver operating characteristics of the *FAIM3:PLAC8* gene expression ratio in discriminating CAP and no-CAP patients. *Horizontal red line* in dot plot denotes the threshold value (0.757). Area under curve (AUC) and 95% confidence interval (CI) analysis was performed by bootstrap resampling (2,000 stratified replicates). *Horizontal black bars* denote medians.

scores, shock, and ICU lengths of stay. Some differences were unearthed for empirical antibiotic treatment, namely cefotaxim and ciprofloxacin (Table 1). Like in the discovery cohort, the most common final diagnoses in no-CAP patients were suspected aspiration, exacerbation of chronic obstructive pulmonary disease or asthma, and congestive heart failure (*see* Table E3). In concordance with microarray discovery data, *PLAC8* gene expression was significantly higher in CAP patients, whereas *FAIM3* gene expression was significantly lower in CAP patients (Figure 4A).The qRT-PCR *FAIM3:PLAC8* ratio showed highly significant discrimination of CAP and no-CAP patients (BH adjusted, $P < 0.0001$).

A numerical threshold was defined at 0.0099 (Figure 4A), again favoring high sensitivity (97.1%) at the expense of specificity (28.6%). At this predefined threshold our qRT-PCR *FAIM3:PLAC8* gene ratio produced 77.2% positive predictive value (95% CI, 73.6–82.1%), 80% negative predictive value (95% CI, 54.5–100%), and 77.6% accuracy (95% CI, 72.5–82.7%). Evaluation of the clinical value by LRs yielded LR+ 1.36, LR− 0.1, and post-test probabilities of being CAP positive or negative were 77% and 20%, respectively. Moreover, threshold-independent AUC analysis yielded 0.784 (95% CI, 0.668–0.886) (Figure 4B). We also explored the performance of the *FAIM3:PLAC8* gene expression ratio in important patient subgroups (*see* Figures E6 and E7). Threshold-dependent LRs and post-test probabilities are tabulated in Table E5. Taking into account that our sample size is too small for such subgroup analyses with appropriate power, these exploratory analyses showed that our candidate *FAIM3:PLAC8* biomarker performed well in these subgroups. Altogether, these findings

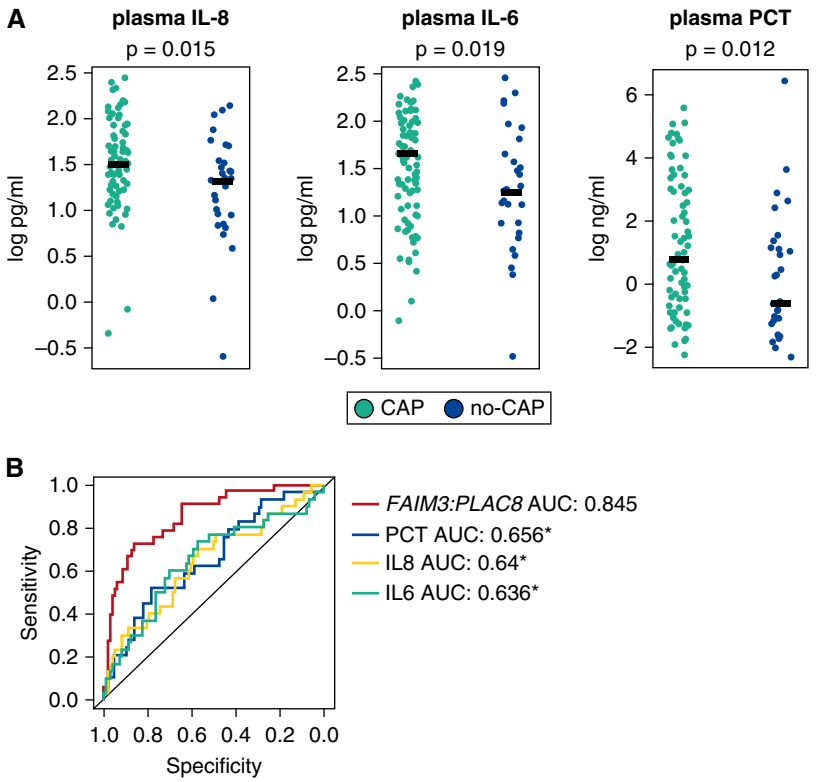

Figure 3. Comparison of *FAIM3:PLAC8* gene expression ratio biomarker and protein biomarkers in consecutively enrolled patients treated for suspected community-acquired pneumonia (CAP) on intensive care unit admission. (*A*) Plasma concentrations of the discovery cohort of CAP and no-CAP (noninfectious control subjects) patients were evaluated for IL-8, IL-6, and procalcitonin (PCT) abundance. Wilcoxon rank sum tests were used to test statistical significance. IL-6, IL-8, and PCT concentrations were significantly different between CAP and no-CAP patients. (*B*) Receiver operating characteristic area under curve (AUC) analysis (2,000 stratified bootstrap replicates) showed protein biomarkers were poorly suited for the discrimination of CAP and no-CAP patients. *AUCs for IL-6, IL-8, and PCT were significantly different ($P < 0.01$) from the gene expression *FAIM3:PLAC8* ratio biomarker AUC. *Horizontal black bars* in dot plots denote medians.

provide robustness to our gene expression classification of CAP and no-CAP patients at ICU admission.

Discussion

Despite the global impact of sepsis and its sequelae, our understanding of the host responses that ensue and their relationship to clinical presentations remains incomplete. Using an unbiased genome-wide blood transcriptional strategy we found that the host leukocyte response to severe CAP presented shared and distinct patterns of transcription as compared with noninfectious critically ill patients that were treated for CAP at ICU admission but *post hoc* were considered to not have pneumonia. We also derived a 78-gene signature for CAP that we refined to a combinatorial qRT-PCR test and propose this candidate blood biomarker for assisting in the rapid diagnosis of CAP at ICU admission.

The complex and multifaceted host reactions underlying the septic response preclude the sole reliance on clinical definitions, which are mainly nonspecific (29). Moreover, reductionist approaches alone cannot capture the higher-order interplay of molecular networks and interactions that constitute a protective response or determine progression to multiple organ failure. To better understand the pathogenesis of sepsis, researchers have probed the whole-blood leukocyte transcriptome in the context of controlled model systems in humans (30, 31) and animals (32, 33). Whole-blood leukocyte transcriptional data in critically ill adult patients with sepsis have also been gathered for diagnostic prediction (20, 34–36), prognostics (37–40), and functional analysis (21, 41). In these studies inclusion criteria varied between post-trauma, burns, systemic inflammatory response syndrome, and sepsis. Control groups were comprised of clinically clearly different subjects, including healthy individuals, postoperative, systemic inflammatory response syndrome, or patients with no sepsis. In contrast to these studies, our study was designed to construct a molecular classifier to enable the rapid discrimination between two groups of critically ill patients who were diagnosed with CAP by treating physicians at ICU admission (Table 1).

Notwithstanding the varying degree of inclusion criteria and control group stratification in those previously published studies, overexpression of proinflammatory and antiinflammatory genes coupled with underexpression of lymphocyte and antigen presentation were common findings. Our discovery cohort of 134 critically ill patients also showed the same patterns of gene expression. Notably, proinflammatory and antiinflammatory genes were similarly overexpressed in both CAP and no-CAP patients suggesting these pathways and others (*see* Figure E2) may constitute a common sickness and/or treatment response. Similar observations of a common host response were noted in previous animal and *ex vivo* human cell studies (42, 43). Thus, besides lending weight to the "common host response" concept in a clinical context, our findings also suggest it is not exclusive to infection but also applies to noninfectious critical illness. The differences between CAP and no-CAP patients were underexpression of EIF2 signaling (RNA translation), mTOR signaling, and T-cell pathways (Figure 1D). Moreover, our CAP gene signature encompassed a number of major histocompatibility class II genes that were underexpressed, including *HLA-DPB1*, *HLA-DPA1*, *HLA-DMA*, and *HLA-DMB* (Figure 2A). These differences provide further evidence for a current paradigm regarding the host response in sepsis, that of immunosuppression (44).

A substantial number of biomarkers have been proposed for the diagnostic stratification of infectious and noninfectious ICU patients (10–12, 45). The most widely used contenders have been C-reactive protein (46), soluble triggering receptor expressed on myeloid cells 1 (47), and

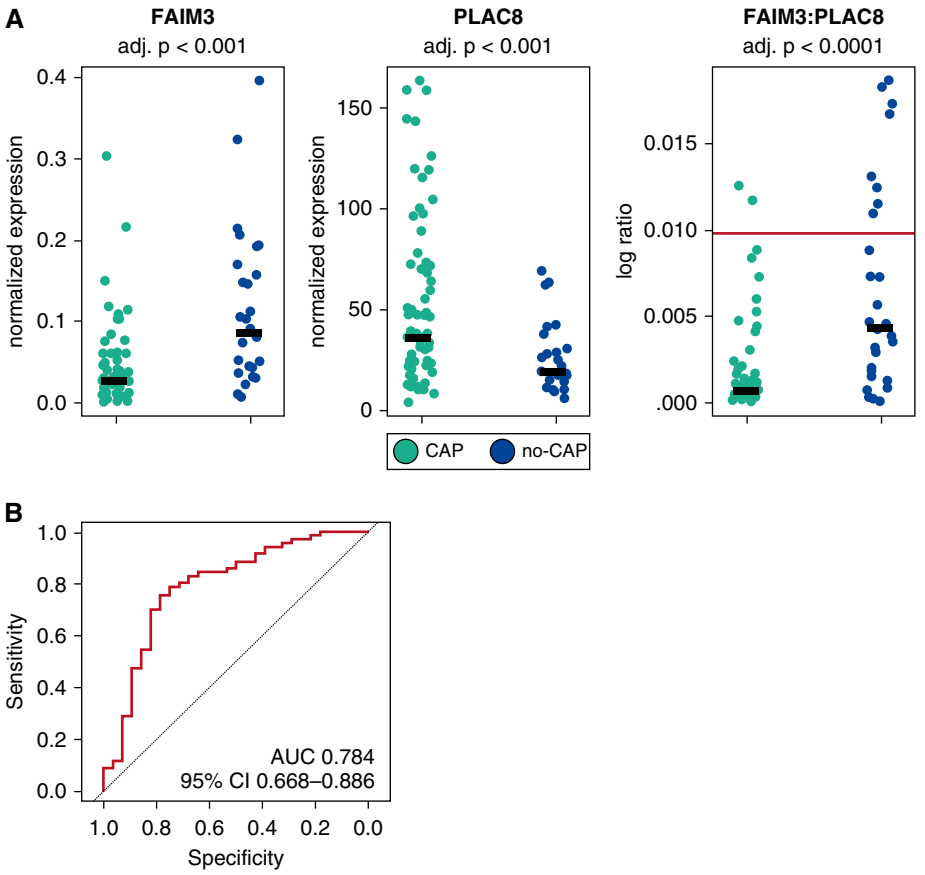

Figure 4. Community-acquired pneumonia (CAP) and noninfectious intensive care unit patients treated for suspected CAP (no-CAP) independent cohort assessment of the *FAIM3*:*PLAC8* gene expression ratio biomarker by quantitative reverse-transcriptase polymerase chain reaction analysis. (*A*) Dot plots of *FAIM3* and *PLAC8* (both normalized to *HPRT1* expression) and *FAIM3*:*PLAC8* log ratio in CAP and no-CAP patients. adj. p = Benjamini-Hochberg adjusted Wilcoxon rank sum test *P* value. *Horizontal red line* in *FAIM3*:*PLAC8* dot plot denotes threshold value (0.0099). *Horizontal black bars* denote medians. (*B*) Receiver operating characteristic area under curve (AUC) and 95% confidence interval (CI) computed by bootstrap resampling (2,000 stratified replicates).

procalcitonin (48). By virtue of metaanalysis, procalcitonin was deemed a more accurate biomarker than C-reactive protein (49), albeit investigations into the accuracy of procalcitonin for the diagnosis of sepsis have provided conflicting results (11, 50, 51). Despite marked heterogeneity in clinical criteria for group stratification, such studies highlighted that the lack of specificity was a major bottleneck. This reflected on the moderate AUCs, where the most recent procalcitonin metaanalysis provided a summary AUC of 0.85 (95% CI, 0.81–0.88) (11).

The heterogeneous etiology of the host response to infection precludes the sole use of one measurement to consistently predict infection among critically ill patients. The 2007 Food and Drug Administration draft guideline on *in vitro* diagnostic multivariate index assays (52) gives precedence to the use of a combination of multiple variables to yield a single, patient-specific result that is intended for use in diagnosis, treatment, mitigation, or prevention of disease. The use of multiple tests in ratio combinations has been routinely practiced by physicians (e.g., risk stratification in cancer) (53–55). Our combinatorial analysis refined the CAP 78-gene expression signature to the most informative gene expression ratio encompassing two negative regulators of apoptosis, *FAIM3*, encoding the Fas apoptotic inhibitory molecule 3, and *PLAC8*, encoding placenta-specific 8 (Figure 2C). Although we provided robustness to our *FAIM3*:*PLAC8* expression ratio by independent cohort qRT-PCR validation, the LR+, LR−, and post-test probabilities (*see* Tables E4 and E5) preclude the use of our proposed test as a stand-alone diagnostic biomarker in critically ill patients presenting with suspected CAP. Indeed, in the setting of ICU admission for suspected CAP, the *FAIM3*:*PLAC8* biomarker does not have a good enough negative predictive value to justify withholding potentially lifesaving antibiotic therapy, a conclusion previously drawn for other sepsis biomarkers in the ICU (13–15).

The use of a diagnostic biomarker for infection in the ICU differs from its use in less severely ill patients, such as mild-to-moderate respiratory tract infection, in whom withholding antibiotics is relatively safe (56). Nonetheless, the *FAIM3*:*PLAC8* biomarker performed better than advocated protein biomarkers, including procalcitonin and IL-6 and -8. In addition, the *FAIM3*:*PLAC8* biomarker might be useful in identifying patients admitted to the ICU with suspected CAP in whom the likelihood of infection is relatively low, thereby urging the clinician to search for alternative diagnoses that may require immediate attention. The fact that the AUCs of protein biomarkers were lower than reported earlier in studies comparing infectious with noninfectious patients (10–12, 45) is likely caused by the design of the current investigation. Using strict diagnostic criteria critically ill patients suspected of having CAP but in retrospect classified as an infection likelihood of "none" by dedicated research physicians were used as case control subjects. Of note, when compared with the discovery cohort, our independent validation cohort presented some differences, especially the APACHE IV severity scores, the presence of shock, and empirical drug treatment (Table 1), which could at least in part be related to seasonal differences. Despite these differences, the *FAIM3*:*PLAC8* gene expression ratio performed well in discriminating CAP and no-CAP patients by qRT-PCR (Figure 4).

We here show that severe CAP altered more than 80% of the normally expressed leukocyte transcriptome. Remarkably, the genomic response of CAP and no-CAP patients was predominantly common and enriched for genes involved in proinflammatory, antiinflammatory, and metabolic signaling pathways. Underexpression of EIF2 signaling, mTOR

signaling, and T-cell pathways was associated with CAP as compared with no-CAP patients. We derived a robust *FAIM3*:*PLAC8* gene expression ratio as a candidate biomarker to assist the clinician in rapidly diagnosing CAP. The predictive performance of the *FAIM3*:*PLAC8* candidate biomarker precludes its sole use in intensive care decision making. The clinical value of this novel biomarker needs to be confirmed in future prospective studies, not only within the context of pneumonia but also considering other sites of infection. A major advantage of a gene expression biomarker derived from whole blood is that it can be readily incorporated in a point-of-care PCR-based test (16, 22). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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