An Evaluation of the Role of Gene Expression in the Prediction and Diagnosis of Ventilator-associated Pneumonia

Julien Textoris, M.D.,* Béatrice Loriod, B.S.,† Laurent Benayoun, M.D.,‡ Pierre-Antoine Gourraud, Ph.D.,§ Denis Puthier, M.D.,¶ Jacques Albanèse, M.D., Ph.D.,# Jean Mantz, M.D., Ph.D.,** Claude Martin, M.D.,†† Catherine Nguyen, Ph.D.,‡‡ Marc Leone, M.D., Ph.D.#

ABSTRACT

Background: The Sepsichip project explored transcriptional modulation associated with ventilator-associated pneumonia (VAP) in patients admitted to the intensive care unit for trauma. Genome-wide expression analysis may help to identify potential diagnostic markers for diseases. The current study examined the changes in blood transcriptome during VAP.

Methods: The authors prospectively included 165 trauma patients, and 41 developed VAP. Whole blood samples were collected at admission and at VAP. To predict VAP, the admission samples were compared by microarray in patients who did or did not develop VAP. To identify diagnosis markers, paired samples of 35 patients who developed VAP were analyzed. Using NanoString (Seattle, WA), the results were confirmed in the patients who developed VAP. Trauma patients who did not develop VAP served as controls to eliminate a time effect.

Results: The injury severity scores of the patients who did or did not develop VAP were 36 and 29, respectively. No predictive biomarker was identified. For patients who developed VAP, a transcriptional signature was identified between the two sampling times. However, this signature was a generalized pattern related to trauma, independent of the infectious process. Genes involved in the proinflammatory response were down-regulated in the patients who developed VAP, but this difference was not statistically significant.

Conclusions: In contrast to clinical assessment, transcriptional analysis of whole blood samples cannot predict or diagnose VAP in trauma patients. Differentiating infection from inflammation seems challenging.

What This Article Tells Us That Is New

- Transcriptional analysis, using DNA microarrays containing 8,682 genes, of 165 trauma patient’s blood was done in patients with (41) and without (42) VAP but could not distinguish inflammation from infection

What We Already Know about This Topic

- The clinical diagnostic criteria for ventilator-associated pneumonia (VAP) do not exclude patients who do not have VAP (true negatives)
matory response, making the diagnosis of ventilator-associated pneumonia (VAP) difficult. Chest imaging often is irrelevant because of contusions. In this setting, clinical scores are not informative.\textsuperscript{3} Thus, biologic markers may have a role in predicting or diagnosing VAP in patients. The concentrations of procalcitonin can reinforce the diagnosis of infection, but its value as a diagnostic tool remains a matter of debate.\textsuperscript{4,5} Several high-throughput studies suggest that genome-wide expression analysis could help with the diagnosis of VAP.\textsuperscript{6}

High-throughput technologies may prove to be helpful for deciphering the complex host response to infection. These technologies may provide tools for predicting the occurrence of infection or facilitating its diagnosis. Using genome-wide expression analysis, the signature profiles of inflammation have been characterized by many groups (e.g., trauma,\textsuperscript{6–9} lipopolysaccharide infusion,\textsuperscript{10} malaria,\textsuperscript{11} septic shock in children,\textsuperscript{12,13} severe sepsis and septic shock in adults,\textsuperscript{6,14–17} and Gram-specific infections\textsuperscript{6,18,19}).

To investigate the host response to infection, we attempted to reduce the background noise of inflammation by using trauma patients who developed VAP as their own controls. We prospectively included trauma patients and collected whole blood samples for transcriptome analysis. We conducted an unbiased analysis by exploring the transcriptome using DNA-microarray technology. We compared samples taken at the time of admission from trauma patients who did or did not develop VAP to identify prognostic markers. We also compared paired samples taken from trauma patients at the time of admission and at the time of diagnosis of VAP to identify potential diagnostic markers of VAP. Finally, to eliminate general gene expression patterns related to the elapse of time after trauma, we collected late samples from control trauma patients who did not develop VAP. To confirm our microarray findings, we analyzed the transcriptome with an alternative technology (NanoString nCounter Analysis System; NanoString Technologies, Seattle, WA). We used this technology to confirm our potential gene expression signature and to investigate the expression of some already known inflammatory markers.

Materials and Methods

Patients
The study protocol was approved by the Ethics Committee (Comité de Protection des Personnes Sud-Méditerranée II, no. 206–005). All trauma patients admitted to two surgical intensive care units (ICU) in teaching hospitals (Hôpital de Beaumont, Clichy, France; Hôpital Nord, Marseilles, France) were screened for inclusion. All trauma patients with an injury severity score greater than 15, who fit the criteria of systemic inflammatory response syndrome, and were between the ages of 18 and 60 yr were invited to participate in the study. They were included after informed consent was signed by their next of kin. Exclusion criteria were pregnancy, immunodeficiency, high-dose corticotherapy (more than 20 mg prednisone or equivalent), or expected death within the first 48 h.

Overall Study Design
The diagnosis of VAP was established when the following criteria were fulfilled: purulent bronchial sputum; body temperature more than 38°C or less than 36°C; leukocytes more than 10\textsuperscript{9}/l or less than 4\textsuperscript{9}/l; chest radiograph showing new or progressive infiltrates; presence of at least one microorganism at a concentration of \( \geq 10^4 \) colony-forming units (cfu)/ml in bronchoalveolar lavage, or \( \geq 10^6 \) cfu/ml in tracheal aspirates.\textsuperscript{20} Senior intensivists and microbiologists made the final diagnosis of VAP.

We defined two sample time points (fig. 1). A blood sample was collected within the first 24 h after ICU admission in all trauma patients fulfilling inclusion criteria (S1). Those who developed VAP were sampled a second time at the onset of antibiotic administration (S2). One hundred thirty-one samples (from 90 patients) were run on microarrays, including 49 S1 samples from patients who did not develop VAP and 41 S1-S2 pairs of samples from patients who developed VAP. Because of technical difficulties, some microarrays were discarded. Therefore, data from 77 patients were analyzed, including 35 paired S1-S2 samples from patients who developed VAP, and 42 S1 samples from patients who did not develop VAP. To exclude a generalized effect related to the elapse of time after trauma, 15 trauma patients who did not develop VAP were sampled between days 5 and 10. Because of a lack of RNA, blood was sampled in 13 additional trauma patients who developed VAP. These 28 samples were used to confirm microarray results. We checked that the two groups of patients analyzed by either microarray (n = 90) or NanoString (n = 28) were similar to the whole population included in the study (see Supplemental Digital Content 1, a table summarizing patients' characteristics for each subgroup, http://links.lww.com/ALN/A756).
Sample and Data Collection
Whole blood samples were collected within 24 h of ICU admission (Paxgene®-PreAnalytiX®; Qiagen/Becton Dickson, Valencia, CA) and at the onset of VAP (fig. 1). Blood was withdrawn from an arterial line directly into the PaxGene® tubes. Mixed samples were stored 8 h at 4°C, 24 h at −18°C, and were then maintained at −80°C until RNA extraction. Clinical and biologic data were collected from patient charts at the time of sample collections using our local web-based database.

RNA Extraction and Labeling
Total RNA was isolated from whole blood samples using the PaxGene® Blood RNA System (PreAnalytiX®) according to the manufacturer’s specifications. RNA quality was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), which analyzes the entire RNA electrophoretic trace to evaluate RNA integrity. RNA was extracted from three independent tubes for each time point. High-quality samples were pooled (for each patient and time point). RNA was quantified by spectrophotometry (NanoDrop®; ThermoFisher Scientific, Waltham, MA). As reported previously, hybridization from whole blood samples collected with the PaxGene® system results in a low percentage of genes termed “present” in the microarray analysis. This was mostly attributable to the high expression levels of a small number of genes in the erythrocytes. To improve the hybridization signals, 15 μg each RNA sample, instead of the 5 μg recommended in the standard protocol, was reverse-transcribed into double-stranded complementary DNA (cDNA) and labeled with P32-cytosine. For each time point, all RNAs of each patient that were of good quality, based on the peak profiles of 18S and 28S ribosomal RNA, were pooled. All labeled cDNA generated from these messenger RNA (mRNA) were of the expected radioactive intensity and were hybridized to the microarrays.

Microarray Design and Hybridization
The microarray (HuSG9 k) was developed in our laboratory (Technologie Avancées pour le Génome et la Clinique [TAGC], INSERM U928, Marseilles, France) as described elsewhere. Briefly, HuSG9 k is a human cDNA library, designed from IMAGE clones, which is arranged on a nylon microarray. The microarray contains 9,216 probes comprising 8,682 genes, 100 duplicated genes, and 434 controls. The microarray design was not enriched in specific pathways. Only genes with known functions were selected. Probes were selected by bioinformatics analysis to match the 3’ end of the major transcripts of each gene (Microarray Quality Control, TAGC, INSERM U928). Polymerase chain reaction (PCR) amplification was performed as described previously, and PCR products were spotted onto nylon membranes (Hybond-N+; GE Healthcare, Little Chalfont, Buckinghamshire, England) with a MicroGrid II arrayer (Affymetrix, Santa Clara, CA). PCR amplification products of selected clones were 1,500 base pairs long on average. The robot used a print head with a 16 × 4 arrangement of print tips. Each tip printed 144 spots in a 12 × 12 grid. Additional details regarding the HuSG9 k microarray are available on the TAGC Web site. Because of the high abundance of erythrocyte-related mRNA species in the samples, certain probes were overexpressed and interfered with analysis of the neighboring probes on the microarray. These overly abundant probes were diluted before being spotted on the microarrays. cDNA preparations, hybridizations, and washes were carried out as described previously.

NanoString nCounter Assay
In several studies, the NanoString nCounter assay has been shown to be a novel, effective alternative to SYBR green real-time PCR. In brief, a multiplexed probe library is made with two sequence-specific probes for each gene of interest. The first probe contains a 35- to 50-base pair sequence, complementary to a particular target mRNA, plus a short common sequence that is coupled to an affinity tag. In our study, the affinity tag is biotin. The second probe contains a second 35- to 50-base sequence, complementary to the target mRNA, which is coupled to an RNA-based, color-coded tag that provides the detection signal. The linear order of these differently colored RNA segments creates a unique code for each gene of interest. Unique pairs of capture and reporter probes are constructed to detect transcripts for each gene of interest. All probes are mixed together with total RNA in a single hybridization reaction that proceeds in solution. After each complex is captured on a surface coated with streptavidin, an applied electric field extends and orients each of them in the solution in the same direction. The complexes are then immobilized in an elongated state and imaged. Each target molecule of interest is identified by the color code generated by the ordered fluorescent segments present on the reporter probe. The level of expression is measured by counting the number of codes for each mRNA. A complete description and performance analysis of the NanoString nCounter assay can be found in the seminal article. In our study, 100 ng each sample total RNA was sent to Seattle’s NanoString laboratories. Samples were collected from a cohort of 28 patients who were not analyzed by microarray. The raw results were normalized using internal controls provided by the manufacturer. Differences in the normalized counts were assessed with the Wilcoxon rank sum test and were called significant if P < 0.05.

Microarray and Statistical Analysis
Microarray image files were captured with a phosphor imager (BAS5000; FujiFilm, Tokyo, Japan). Data from .INF or .IMG files were extracted with BZScan2 software (TAGC, INSERM U928). Data files were loaded and analyzed with R and Bioconductor, using the NylonArray library deve-
Table 1. Patient Characteristics at Admission

<table>
<thead>
<tr>
<th></th>
<th>Patients Who Did Not Develop VAP (n = 124)</th>
<th>Patients Who Developed VAP (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>31 [23–41]</td>
<td>30 [22–46]</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 [23–25]</td>
<td>25 [23–26]</td>
</tr>
<tr>
<td>Head trauma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (%)</td>
<td>104 (84)</td>
<td>38 (92)</td>
</tr>
<tr>
<td>Chest trauma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (%)</td>
<td>57 (46)</td>
<td>28 (68)*</td>
</tr>
<tr>
<td>AIS</td>
<td>3 [0–3]</td>
<td>3 [2–4]</td>
</tr>
<tr>
<td>Glasgow Coma Scale</td>
<td>7 [4–11]</td>
<td>5 [3–8]</td>
</tr>
<tr>
<td>SAPS II</td>
<td>41 [36–51]</td>
<td>47 [43–53]</td>
</tr>
<tr>
<td>Injury Severity Score</td>
<td>29 [24–37]</td>
<td>36 [26–43]*</td>
</tr>
<tr>
<td>Heart rate (/min)</td>
<td>116 [100–129]</td>
<td>114 [99–124]</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>96 (77)</td>
<td>38 (92)</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>37.9 [36.1–38.7]</td>
<td>38.7 [36.2–39.2]</td>
</tr>
<tr>
<td>Creatinine (µM)</td>
<td>81 [70–97]</td>
<td>73 [57–90]</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>10.5 [8.9–12.5]</td>
<td>10.3 [8.7–11.5]</td>
</tr>
<tr>
<td>Platelets (g/l)</td>
<td>178 [135–275]</td>
<td>172 [123–218]</td>
</tr>
<tr>
<td>Fibrinogen (mM)</td>
<td>2.4 [1.9–3]</td>
<td>4.5 [2.5–6.6]*</td>
</tr>
<tr>
<td>PaO₂/FiO₂ ratio</td>
<td>282 [268–345]</td>
<td>218 [195–287]</td>
</tr>
<tr>
<td>Plasma lactate (mM)</td>
<td>1.9 [1.4–3.5]</td>
<td>1.6 [1.2–2]</td>
</tr>
<tr>
<td>Death n (%)</td>
<td>24 (19)</td>
<td>11 (27)</td>
</tr>
</tbody>
</table>

Data are presented as median [interquartile range] or as absolute count (percentage).

* P < 0.05.

AIS = Acute Injury Score; BMI = body mass index; SAPS = Simplified Acute Physiological Score; VAP = ventilator-associated pneumonia.

Results

Characteristics of Patients

A cohort of 165 trauma patients was prospectively included in the study. Of these, 41 (25%) patients developed VAP. All patients had severe multiple trauma involving the head (86%), chest (52%), abdomen (36%), and limbs (48%). At the time of admission, the injury severity score was higher in the trauma patients who developed VAP than in those who did not develop VAP (median [interquartile range]: 36 [26–43] vs. 29 [24–37], P = 0.04). At the thoracic level, an acute injury score greater than 2 was found in 28 (68%) patients who developed VAP, compared with 57 (46%) patients who did not develop VAP (P = 0.02) (table 1). Death occurred in 11 (27%) patients who developed VAP and in 24 (19%) patients who did not develop VAP (P = 0.42). Median onset day of pneumonia was day 8. Clinical and laboratory variables of patients who developed VAP are provided in table 2. The clinical and laboratory characteristics of these patients were similar, except for PaO₂, which was lower at the onset of VAP.

No Transcriptional Signature Predicts VAP in Trauma Patients

To identify prognostic markers of VAP, we compared samples collected at the time of ICU admission from trauma patients who developed VAP and those who did not develop VAP. After we excluded hybridizations because of technical difficulties, 39 admission samples from patients who developed VAP were compared with 42 admission samples from patients who did not develop VAP. The expression profile was similar in the trauma patients who developed VAP and
Differential Gene Expression between Admission Time and Onset of VAP in Trauma Patients

To identify diagnostic markers of VAP, we compared admission samples from trauma patients who developed VAP with samples collected in the same patients at the onset of VAP. After the exclusion of six hybridizations (because of technical issues), we analyzed 35 paired samples.

The analysis of normalized gene expression identified 207 probes whose expression levels differed between the two sampling times (false discovery rate less than 10%). The heat map shows this signature by representing, according to a color code, the expression value of each gene. The heat map shows this signature by representing, according to a color code, the expression value of each gene.

43 probes whose expression levels differed between the two sampling times (false discovery rate less than 1%). The heat map shows this signature by representing, according to a color code, the expression value of each gene.

Table 2. Characteristics of Patients Who Developed Ventilator-associated Pneumonia

<table>
<thead>
<tr>
<th></th>
<th>Admission (n = 41)</th>
<th>Onset of VAP (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (/min)</td>
<td>108 [95–124]</td>
<td>114 [99–128]</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>38.4 [36.3–39.2]</td>
<td>38.5 [36.1–38.7]</td>
</tr>
<tr>
<td>Creatinine (μM)</td>
<td>77 [69–99]</td>
<td>65 [54–75]*</td>
</tr>
<tr>
<td>Glycemia (mmol/l)</td>
<td>6.5 [5.8–7.3]</td>
<td>6.4 [5.8–7.3]</td>
</tr>
<tr>
<td>Leukocytes (g/l)</td>
<td>13 [10–17]</td>
<td>13 [10–18]</td>
</tr>
<tr>
<td>Plasma lactate</td>
<td>1.8 [1.2–2.6]</td>
<td>1.5 [1.2–1.8]</td>
</tr>
<tr>
<td>PaO2 (mmHg)</td>
<td>135 [96–175]</td>
<td>91 [79–110]*</td>
</tr>
<tr>
<td>FiO2 (%)</td>
<td>48 [40–56]</td>
<td>50 [40–60]</td>
</tr>
<tr>
<td>SacO2 (%)</td>
<td>99 [98–99]</td>
<td>98 [97–99]</td>
</tr>
</tbody>
</table>

Clinical and biological characteristics of the patients who developed a ventilator-associated pneumonia (VAP), at admission and at the onset of VAP. Data are presented as median [interquartile range].

* P < 0.01.

Table 3. Inflammatory Modulation between Admission and Onset of VAP

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Change Microarray</th>
<th>Fold Change NanoString</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1B</td>
<td>0.80</td>
<td>0.40</td>
</tr>
<tr>
<td>IL1RN</td>
<td>0.60</td>
<td>0.57</td>
</tr>
<tr>
<td>IL6</td>
<td>1.03</td>
<td>—</td>
</tr>
<tr>
<td>IL6R</td>
<td>0.93</td>
<td>0.77</td>
</tr>
<tr>
<td>HMGB1</td>
<td>0.77</td>
<td>0.86</td>
</tr>
<tr>
<td>TREM1</td>
<td>0.87</td>
<td>0.70</td>
</tr>
<tr>
<td>ICAM1</td>
<td>0.95</td>
<td>0.97</td>
</tr>
<tr>
<td>TNF</td>
<td>—</td>
<td>0.70</td>
</tr>
<tr>
<td>CALCA</td>
<td>1.21</td>
<td>—</td>
</tr>
<tr>
<td>CALCB</td>
<td>1.23</td>
<td>—</td>
</tr>
<tr>
<td>CRP</td>
<td>1.01</td>
<td>—</td>
</tr>
</tbody>
</table>

Microarray fold changes are presented for patients who developed VAP. NanoString fold changes are presented for patients who developed VAP and controls. Eleven markers were tested. TNF was not tested in microarray. Four genes were not expressed in NanoString nCounter assay: IL6, CALCA, CALCB, and CRP.

CALCA = calcitonin-related polypeptide alpha; CALCB = calcitonin-related polypeptide beta; CRP = C-reactive protein; HMGB1 = high mobility group box 1; ICAM1 = intercellular adhesion molecule 1; IL1B = interleukin 1 beta; IL1RN = interleukin 1 receptor antagonist; IL6 = interleukin 6; IL6R = interleukin 6 receptor; TNF = tumor necrosis factor; TREM1 = triggering receptor expressed on myeloid cells 1; VAP = ventilator-associated pneumonia.

Confirmation of the Selected Markers with the NanoString nCounter Technology

The NanoString nCounter technology was used to validate the expression changes of the 10 genes that are potential diagnostic markers of VAP. We used samples from a new cohort of 13 trauma patients who developed VAP. Results with the NanoString nCounter assay confirmed the modulation of five (ALAS2, AHSP, SPARC, PPBP, and PCSK1) of the 10 selected genes (fig. 2A). For these five genes, we found a statistically significant up-regulation in the time between admission and onset of VAP.

To exclude a generalized effect on gene expression related to trauma and not VAP, we analyzed samples from 15 trauma patients who did not develop VAP. These patients were free of infection and antibiotic treatment. These samples...
were collected between days 5 and 10. The signature of 10 genes was tested in these samples. A similar profile of expression was found, including the five selected genes (fig. 2A).

**Decrease of Proinflammatory Gene Expression Is Associated with VAP**

We also tested a set of already known inflammatory genes: TREM1, TNF, IL6R, IL6, IL1B, IL1RN, ICAM1, HMGB1, CRP, CALCA, and CALCB. Not enough signal was detected for IL6, CRP, CALCA, and CALCB. These four genes were excluded from the analysis. With the use of NanoString nCounter technology, IL1B, IL1RN, TNF, IL6R, and TREM1 were down-regulated in samples from patients at the onset of VAP. In addition, these five proinflammatory markers differentiated patients who developed VAP and those who did not develop VAP (fig. 2B).

**Discussion**

Using a whole blood transcriptome analysis, the SepsisChip project was designed to identify a signature of gene expression associated with the development of VAP. Among 165 multiple trauma patients, 41 developed VAP. As previously described, we confirmed an association between the development of VAP and the severity of trauma. Despite these clinical differences, the transcriptome analysis does not show any difference between trauma patients who did or did not develop VAP. Therefore, this analysis cannot serve to predict the clinical outcome of trauma patients. Comparison of samples at the time of admission and at the time of VAP onset identifies a 207-gene transcriptional signature. These genes are involved in basal cell functions, such as transcription, translation, apoptosis, immune response, hemostasis, and coagulation. Of note, genes involved in the proinflammatory response are nonsignificantly down-regulated. Focusing on genes with a false discovery rate less than 1%, we identified a 10-gene signature for the potential diagnosis of VAP. Using NanoString, we confirmed the up-regulation of 5 of these 10 genes. However, we found a similar signature in a cohort of trauma patients who did not develop VAP, suggesting a generalized effect related to trauma. In an additional analysis, we identified a down-regulation of proinflammatory mediators only in the patients who developed VAP.

In contrast to experimental studies, clinical investigations often are limited by excessive heterogeneity of the included patients. To reduce this bias, we selected trauma patients in a predetermined age range. We also used a single model of infection. Because genetic backgrounds can differ among patients, we constructed a paired analysis. In our study, analysis of samples from the same patient at admission and at onset of VAP serves as a control for genetic heterogeneity. This design also let us compare the host response to inflammation (trauma) and infection (VAP). This may improve the characterization of genes modulated by infection, independently of inflammation. In agreement with the findings of previous
studies, classic markers of the inflammatory response in our study were up-regulated at admission. This early and transient proinflammatory response was also found in a murine model of acute lung injury. A signature of 207 probes discriminates the samples of patients at the time of admission and at the onset of VAP. Actually, we found that this signature is related to a generalized response after trauma. Thus, no specific signature of infection is found in trauma patients who developed VAP.

This striking finding shows that the transcriptional changes observed in the trauma patients are caused by inflammation and other general effects after trauma. In other studies reporting signatures for severe sepsis, the controls are healthy volunteers. Thus, these studies cannot differentiate infection and inflammation. The signatures that were reported in these previous studies may be related to inflammation and not specifically to the infectious process.

In a seminal paper, McDunn et al. suggest that the onset of an infection-specific transcriptional program may precede the clinical diagnosis of VAP. Using a bench-to-bedside approach, 85 genes were modulated during the 7 days bracketing the diagnosis of VAP. A general trajectory (entitled “riboleukogram”) was identified. In our study, we tested 52 of these 85 genes. Forty-one (79%) genes exhibited a similar modulation. In light of our findings, the trajectories associated with the riboleukogram may be related to inflammation more than infection. However, the concept of a general trajectory may be more useful diagnostically than a single-point analysis.

Obtaining high-quality genomic data from critically ill patients remains a challenge. Cell collection methods, mRNA extraction, and labeling methods influence the expression profiles. Buffy coat versus whole blood sampling is discussed elsewhere. As described previously, whole blood samples resulted in a weak hybridization signal. Because a few probes exhibited overexposed signals in preliminary experiments (data not shown), we changed our protocols by diluting these probes on all microarrays. Next, we increased the quantity of RNA and P33-cytosine for the cDNA preparations. These changes corrected the percentage of present calls into a normal range. These issues are related to the use of whole blood samples. However, both its reproducibility and its simplicity in the setting of a multicenter clinical study drive the choice of Paxgene.

Our microarray findings were confirmed by using the novel NanoString nCounter Analysis System. NanoString uses digital technology based on direct multiplexed measurement of gene expression. It offers a high level of sensitivity, precision, and reproducibility. NanoStrings are fluorescent bar codes that bind to target mRNA for gene expression analysis. The advantages are its sensitivity comparable with PCR, the lack of enzymology or amplification, and the ability to analyze the expression levels of as many as 800 genes in a single assay. Using this system, we confirm the modulation observed by microarray analysis for 5 of the 10 gene of the signature.

To exclude a general effect after trauma, we tested these five potential biomarkers in a set of patients who did not develop VAP. Indeed, the inflammatory profile of trauma patients is affected by different procedures, such as mechanical ventilation, parenteral nutrition, and surgical procedures. In this cohort, the five genes are up-regulated, despite that these patients did not develop VAP. Thus, these genes are not specific for an infectious process. We also tested a set of markers that are classically involved in inflammation and infection. Five proinflammatory mediators are down-regulated in the patients who developed VAP, whereas they are unchanged in our controls. The same down-regulation is also detected in the microarray data.

This down-regulation in patients who develop VAP is of interest because the polarization of the immune response in ICU patients remains a matter of debate. This supports the concept of immunosuppression in ICU patients with infection. As highlighted elsewhere, the immune system of septic patients is unable to respond to a second challenge of endotoxin. This state of nonresponsiveness is known as “anergy.” Autopsy studies in persons who died of sepsis showed a profound, apoptosis-induced loss of adaptive immune system cells. Notably, most studies aimed at inhibiting proinflammatory cytokines failed to improve survival. In contrast, stimulating the immune response in selected immunodeficient septic patients, by granulocyte/macrophage-colony stimulating factor, for example, may improve their outcome. In the current study, we found a decreased expression of TNF, IL1B, IL1RN, IL6, and TREM1 in the patients who developed VAP. Therefore, we confirm the association between a relative immunodeficiency and the sepsis occurrence. This suggests the need to monitor the immunologic status of trauma patients.

Our study has several limitations. It is possible that the heterogeneity of patients included in the study precludes the identification of a positive effect. However, our patients were composed of a relatively homogeneous population of patients with multiple traumas, including 80% with a head injury. To reduce the effect of age, our inclusion criteria were restrictive. Our endpoint was restricted to the development of VAP. Except for seven positive urine cultures, for which the rate of occurrence was similar in patients who did or did not develop VAP, no infection interfered with VAP. The initial analysis, designed to compare the transcriptome at the time of admission for trauma patients who did or did not develop VAP, was negative. Even with a high false discovery rate (20%), we did not find any differentially expressed genes. Thus, we interpreted this result as the absence of prognostic biomarkers of VAP in trauma patients. We also conducted subgroup analysis to infer the potential effect of sex, age, or other infection on transcriptome modulation. No effect was found, but these results (data not shown) should be interpreted with caution because of low power estimates.
Because the blood can be described as a black box, one can suggest that it may not reflect the transcriptional modulation in the lung. Analysis of whole blood from trauma patients most likely does not reflect transcriptional changes in the lung. To address this, we have studied time-related changes in the lung using an animal model of lung injury.

Conclusion

In conclusion, we explored the transcriptome of whole blood samples from 165 trauma patients, 41 of whom developed VAP. Our goal was to identify a gene expression profile that could be used to identify trauma patients at risk for VAP. At the time of admission, the trauma patients who developed VAP had a higher severity score than did those who did not develop VAP. In addition, we found that chest trauma is a risk factor for VAP. Within the limitations of our study, for patients that developed VAP, a transcriptional signature was identified that reflected differential gene expression at time of admission and time of VAP diagnosis. This signature is attributed to a generalized pattern related to trauma and seems independent of the infectious process. Interestingly, five proinflammatory markers are down-regulated at the onset of VAP, although the differences are not statistically significant. They are expressed at a steady level in the patients who did not develop VAP. These results support the concept that infection in the ICU results from an unbalanced shift toward an antiinflammatory state. The trajectory of the inflammatory balance in trauma patients at clinical risk of VAP should be assessed in future studies.

This work is dedicated to Antoine Secondi (Chief Nursing Officer), our friend. Antoine taught us to care, a job in which he excelled; he died the day before this work was accepted. The authors are indebted to Dominique Lemoine, C.R.N.A., Frédéric Navas, C.R.N.A., Véronique Paone, C.R.N.A., Marie-Hélène Po, C.R.N.A., and Antoine Secondi C.R.N.A. (Hôpital Nord, Assistance Publique - Hôpitaux de Marseille, Marseille, France), for the management of samples; Khadja Hammi (Clinical Research Assistant, Assistance Publique - Hôpitaux de Marseille) and Nathalie Leavre, M.D. (Centre d’Investigation Clinique, Hôpital Nord, Marseille, France), for case report form completion and administrative tasks; and François Antonini, M.D. (Service d’Anesthésie et de Réanimation, Hôpital Nord, Assistance Publique - Hôpitaux de Marseille, Université de la Méditerranée, Marseille, France), and Pascal Rihet, Ph.D. (U958, Université de la Méditerranée), for useful comments on the statistical approach.

References


Copyright © by the American Society of Anesthesiologists. Unauthorized reproduction of this article is prohibited.


