Published in final edited form as:

Intensive Care Med. 2013 August ; 39(8): 1423–1434. doi:10.1007/s00134-013-2935-7.

Metabolomics in pneumonia and sepsis: an analysis of the GenIMS cohort study

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Electronic supplementary material: The online version of this article (doi:10.1007/s00134-013-2935-7) contains supplementary material, which is available to authorized users.

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Abstract

Purpose—To determine the global metabolomic profile as measured in circulating plasma from surviving and non-surviving patients with community-acquired pneumonia (CAP) and sepsis.

Methods—Random, outcome-stratified case–control sample from a prospective study of 1,895 patients hospitalized with CAP and sepsis. Cases ($n = 15$) were adults who died before 90 days, and controls ($n = 15$) were adults who survived, matched on demographics, infection type, and procalcitonin. We determined the global metabolomic profile in the first emergency department blood sample using non-targeted mass-spectrometry. We derived metabolite-based prognostic models for 90-day mortality. We determined if metabolites stimulated cytokine production by differentiated Thp1 monocytes in vitro, and validated metabolite profiles in mouse liver and kidney homogenates at 8 h in cecal ligation and puncture (CLP) sepsis.

Results—We identified 423 small molecules, of which the relative levels of 70 (17 %) were different between survivors and non-survivors ($p < 0.05$). Broad differences were present in pathways of oxidative stress, bile acid metabolism, and stress response. Metabolite-based prognostic models for 90-day survival performed modestly (AUC = 0.67, 95 % CI 0.48, 0.81). Five nucleic acid metabolites were greater in non-survivors ($p < 0.05$). Of these, pseudouridine increased monocyte expression of TNF α and IL1 β versus control ($p < 0.05$). Pseudouridine was also increased in liver and kidney homogenates from CLP mice versus sham ($p < 0.05$ for both).

Conclusions—Although replication is required, we show the global metabolomic profile in plasma broadly differs between survivors and non-survivors of CAP and sepsis. Metabolite-based prognostic models had modest performance, though metabolites of oxidative stress may act as putative damage-associated molecular patterns.

Keywords

Sepsis; Pneumonia; Metabolomics; Biomarker

Introduction

Targeted, precise care of patients with sepsis will require a better understanding of mechanisms underlying response to infection and accurate methods to prognosticate sepsis syndromes. Despite decades of work, many sepsis patients go unrecognized by healthcare professionals and thus fail to receive recommended treatment [1, 2]. Given the high, and

potentially rising, incidence of sepsis [3], even small improvements in recognition and tailored treatment may save many lives.

To this end, hundreds, if not thousands, of diagnostic and prognostic biomarkers are proposed in sepsis [4]. These include markers from a variety of biofluids and organs that may indicate the level of activation of the innate immune response [5]. Yet currently, few markers, if any, successfully guide sepsis treatments [6], with notable failures [7].

To better link new therapies with sepsis mechanisms, the focus of biomarker discovery is increasingly directed towards molecular expression profiles, including gene and protein expression [8]. Furthest downstream in the biologic system, however, are small metabolites—such as amino acids, carbohydrates, or lipids. These metabolites may offer a more relevant, amplified signature in sepsis and can be measured using techniques such as ^1H nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) [9]. To date, the application of metabolomic (or metabonomic) profiling in sepsis is limited to animal studies [10–13], or to specific metabolic pathways, such as tryptophan/kynurenine [14], arachidonic acid [15], or arginine metabolism [16], among others [17]. One study found using ^1H -NMR found differences in glutathione, adenosine, sphingomyelin, and phosphatidylserine between healthy controls and ventilated patients with sepsis-associated acute lung injury [18]. However, a difference in metabolomics between healthy individuals and those with critical illness is not surprising. The next step is to determine if, among patients presenting with a serious infection, metabolomics may help identify those destined to fare poorly from those who will recover.

In a preliminary study, we sought to evaluate the global metabolomic differences between patients who did and did not survive at 90 days after CAP hospitalization. We randomly matched patients with extreme differences in outcomes to best uncover metabolomic differences. Our approach was agnostic to avoid restricting analysis to specific pathways or previously published data. We explored the biological significance of novel metabolite markers using in vitro cytokine production after monocyte stimulation, and validated our findings in a mouse liver and kidney from a cecal ligation and puncture sepsis model. Finally, we examined performance of metabolite-based prognostic models.

Methods

Study design, patients, and setting

We chose an outcome-dependent, stratified random sample of subjects drawn from the Genetic and Inflammatory Markers of Sepsis (GenIMS) cohort study [19]. Further details about GenIMS are provided in the Online Resource. Briefly, this cohort included adults (age >18 years) presenting to 28 emergency departments between 2001 and 2003 with a clinical diagnosis of pneumonia and infiltrate on chest radiograph. Informed consent was obtained from the patient or a proxy, and the study was approved by the Institutional Review Boards of the University of Pittsburgh and all participating sites.

Of the 2,320 subjects enrolled, 2,032 were admitted to hospital, and 137 were subsequently excluded because CAP was ruled out. Thus, the final inpatient sample was 1,895. We randomly selected a subset stratified by survival at 90 days. We selected 15 cases (died) and 15 controls (survivors) matched on the following characteristics: (1) age (within 5 years), (2) sex (male), (3) race (white), (4) initial procalcitonin (PCT) >0.25 mg/mL because higher PCT levels are suggestive of bacterial infection [20], (5) CAP organism Gram stain (positive, negative, or unknown), (6) blood sample obtained at the time of ED presentation, and (7) no prior freeze/thaw cycle on blood sample.

Study procedures and blood sampling

Detailed baseline and clinical information was recorded from the subject or proxy, bedside assessments by research nurses, or the medical record. The clinical data were stripped of identifying information and merged with blood sample data. Blood samples were drawn immediately following enrollment into pyrogen-free tubes containing heparin or citrate, and plasma separated by centrifugation into 1.5-mL tubes. Samples were stored frozen at -80°C until assayed, and shipped on dry ice to Metabolon, Durham, NC, USA, for analyses.

Metabolomic profiling

We performed metabolomic profiling using a previously described platform [21]. Metabolites were identified using a mass spectrometry (MS)-based approach that included: (1) sample preparation and metabolite extraction, (2) detection, (3) spectral analysis, (4) normalization and imputation of missing values, (5) visualization of normalized data, and (6) delineation of class-specific metabolomics signatures. See the Online Resource (Fig. e1) for more details.

All samples were accessioned into the Metabolon LIMS system and prepared by methanol extraction to remove protein fractions. The resulting extract was divided into fractions for subsequent UHPLC/MS/MS (positive and negative ionization modes) and GS/MS analysis. Following the appropriate sample preparation techniques, we separated aliquots for UHPLC/MS/MS analysis using a Waters Acquity UPLC and analyzed using an LTQ mass spectrometer. We separated derivatized samples for GC/MS on a 5 % phenyl dimethyl silicone column with helium as the carrier gas, and analyzed samples on a Thermo-Finnigan Trace DSQ mass spectrometer.

We identified metabolites by automated comparison of the ion features from the experimental samples to a reference library of chemical standard entries that included retention time, mass (m/z), preferred adducts, and in-source fragments as well as associated MS spectra. They were curated by visual inspection for quality control using software developed at Metabolon [22]. To assist with data visualization, we rescaled raw area counts for each metabolite by dividing all sample values by the median value for each individual metabolite. We assumed any missing values to be below the limits of detection and these values were imputed with the compound minimum.

To visualize how metabolite expressions deviated from controls, we used z-score analysis to scale each metabolite according to the mean and standard deviation of the control samples. Metabolomic patterns were also visualized using heat maps generated from unsupervised clustering of samples within each group (cases or controls) with a fixed metabolite order. Data was median-centered, per metabolite, and a green/red color scheme was used to indicate a decrease/increase in a metabolite relative level.

Other cytokine assays

We measured interleukin (IL)-6, IL-10, and tumor necrosis factor using an automated immunoassay analyzer (IMMULITE System; Diagnostic Products, Los Angeles, CA, USA). We used the following upper limits of the normal range for each cytokine based on the manufacturer's specifications for each assay: IL-6, 5.9 pg/mL; IL-10, 9.1 pg/mL; and tumor necrosis factor, 8.1 pg/mL. All laboratory personnel were blinded to clinical information.

In-vitro monocyte stimulation

Among significant metabolites ($p < 0.05$, $q < 0.1$) in our agnostic approach, we identified five nucleic acid metabolites (beta-pseudouridine, 1-methyladenosine, *N*-2-methylguanosine, *N*-6-carbamoylthreonyladenosine, allantoin) for in vitro monocyte

stimulation experiments. We hypothesized that these nucleic acid metabolites may function as damage-associated molecular patterns, and stimulate TNF α and IL1 β similar to HMGB-1 and LPS [23]. See the Online Resource for reagent details, and discussion of dose selection. We cultured THP-1 cells (American Type Cell Collection, Manassas, VA, USA) in suspension in RPMI1640 media (Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 100 μ M sodium pyruvate (all from Gibco). To differentiate the monocytes, 0.33×10^6 cells/mL were suspended in media and treated with 5 ng/mL PMA and then plated. After 48 h, adhered cells were washed three times in PBS and then treated for 16 h with compounds of interest at concentrations described. All compounds were dissolved in endotoxin-free water. We measured endotoxin at 61.5–153.8 pg per 250 μ L treatment of 10 mM dose beta-pseudouridine, compared to 4,000 pg/mL in LPS-positive control. After 16 h media was collected and TNF α and IL1 β levels determined by ELISA (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Experiments were repeated after treatment with viral inhibitory peptide of TLR4 (VIPER) and polymyxin B (PMB).

Cecal ligation and puncture sepsis model

Animal protocols were approved by the University of Pittsburgh Institution Animal Care and Use Committee. The experiments were performed in adherence to the National Institutes of Health Guidelines on the use of laboratory animals. Cecal ligation and perforation (CLP) was performed on C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME, USA) aged 6–8 weeks and weighing 20–25 g. These animals were anesthetized with pentobarbital (70 mg/kg IP). A 1- to 2-cm mid-line laparotomy was performed and the cecum identified. Stool was then milked to the tip of the cecum and it was subsequently ligated 1 cm from the tip with a 2–0 silk tie. The cecum is then perforated with a 22G needle and returned into the abdomen. The muscular and skin was closed with a running 2–0 silk suture. Sham animals underwent laparotomy and bowel manipulation without ligation or perforation (also referred to in this text as sham CLP). Mice were sacrificed 8 h post-CLP, the circulation was flushed with cold PBS, and liver and kidney were harvested and snap frozen. No antibiotics were utilized and animals had free access to food and water pre- and post-operatively. Frozen liver and kidney were prepared for analysis using Metabolon's standard solvent extraction method. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS/MS platforms.

Statistical analysis

All statistical analyses were performed using STATA 11.0 (College Station, TX, USA) or the program R (<http://cran.r-project.org/>). We used the Wilcoxon signed rank test and paired Student's *t* test to evaluate if baseline and pre-infection characteristics were similar across matched pairs. To compare cases and controls for levels of metabolites, we log-transformed the relative observed concentrations for each metabolite, and used Wilcoxon signed rank tests, assuming statistical significance at $p = 0.05$. We estimated false discovery rates (FDR) for each metabolite using *q* values to account for multiple comparisons [24]. Only metabolites with $q < 0.1$ were selected for illustration. The ability of metabolites to serve as classifiers of case/control status was determined using random forest models. We created random forests using a supervised classification technique based on an ensemble of decision trees ($n = 50,000$). The set of classification trees was based on continual sampling of subjects and metabolites, and each subject was classified based on the majority votes from all classification trees. We summarized the operating characteristics of these models using sensitivity, specificity, and the area under the receiver operating characteristic curve with 95 % confidence intervals.

Results

Study population

Per our matching technique, the demographics pre-illness characteristics were similar between survivors and deaths at 90 days (Table 1). The proportion of patients with >0 Charlson comorbidities and smoking status were also similar. As expected, subjects who died had greater illness severity measures at ED presentation, such as pneumonia severity index and APACHE III score ($p = 0.02$). Initial plasma IL-6 and TNF were higher in cases, and IL-10 lower, but not significantly different from controls ($p = 0.15$ – 0.96). We also observed, as expected, that organ failure was more common in patients who died from pneumonia and sepsis than in those who survived at 90 days ($p < 0.01$).

Global metabolomic profile

Figure 1 shows the global metabolomic profile for cases compared to controls, with median-scaled metabolite levels. The broad differences between cases and controls span multiple processes and metabolic pathways, including changes in amino acid, nucleotide, energy, bile acid, and sterol metabolism. We identified 423 named metabolites, of which 70 (17 %) were significantly different between cases and controls ($p \leq 0.05$). Of these, 56 metabolites were below a priori thresholds for false discovery rate ($q < 0.1$). The z-score plot demonstrates the magnitude of metabolite differences in sepsis deaths versus survivors (Fig. 2), when normalized area counts were scaled to the control median and standard deviation.

Specific metabolic pathways

Metabolites differed significantly between groups in many notable and complex pathways (Table 2 for $p \leq 0.01$, Online Resource Table e1 for $0.01 < p \leq 0.05$). Consistent with early cholestasis in sepsis, we observed greater relative levels of sulfated bile acids (taurochenolate sulfate and glycochenolate sulfate; $p < 0.01$ for both) and primary bile acids (taurocholate; $p < 0.05$) in plasma of cases versus controls. Differences in steroid metabolism were present, with significantly greater cortisol ($p < 0.01$), cortisone ($p < 0.05$), and sulfated hormones suggestive of increased storage (5- α -pregnan-3 α , 20- β -diol disulfate, 21-hydroxypregnenolone disulfate, pregnenolone sulfate, and pregnanediol-3-glucuronide; $p < 0.04$ for all). We also found evidence of oxidative stress in purine metabolic pathways (Fig. 3). We observed similar levels of xanthine and urate ($p = 0.68$ and 0.36 , respectively), but greater allantoin—a compound produced by free radical oxidation of uric acid ($p < 0.01$). Modified nucleosides, also liberated during oxidative stress, were elevated in cases compared to controls [N_2 , N_2 -dimethylguanosine ($p < 0.01$), N_6 -carbamoylthreonyladenosine ($p < 0.05$), pseudouridine ($p < 0.05$), and N_1 -methyladenosine ($p < 0.01$)].

Metabolite-based classification models

We used the 423 identified plasma metabolites to build prognostic models for death at 90 days among patients with CAP and sepsis at ED presentation. Random forest models demonstrated modest sensitivity of 0.67 (95 % CI 0.39, 0.87) and specificity of 0.67 (95 % CI 0.39, 0.87). The area under the receiver operating characteristic curve for the random forest model was 0.67 (95 % CI 0.48, 0.81).

Monocyte stimulation by nucleic acid metabolites

We sought to determine whether specific metabolites of nucleic acids, identified in our study at increased levels in non-survivors, could stimulate inflammatory cytokine responses in monocytes in vitro [23]. We treated differentiated Thp-1 human monocytes for 16 h with beta-pseudouridine, 1-methyladenosine, N -2-methylguanosine, N -6-

carbamoylthreonlyadenosine, or allantoin and compared TNF α and IL-1 β levels in cell culture supernatants with levels in supernatants from cells treated with PBS only (control), a known DAMP (HMGB-1) or LPS. We found that beta-pseudouridine, a pyrimidine nucleotide, stimulated significant release of TNF α and IL-1 β in supernatants, and allantoin also significantly increased IL-1 β production but at lower levels (Fig. 4a, b; $p < 0.05$). Cytokine stimulation by beta-pseudouridine was concentration-dependent and greatest at higher metabolite concentrations (Fig. 4c). Cytokine levels remained significantly elevated in the presence of endotoxin-binding agent, polymyxin B, but were significantly abrogated in the presence of TLR4-receptor inhibitor (TLR4 inhibition by viral inhibitory peptide—VIPER) (Fig. 4d).

Cecal ligation and puncture mouse model

We observed a significant relative difference in both liver and kidney homogenate at 8 h after CLP compared to sham in pseudouridine ($p < 0.05$, $q < 0.05$ for both; Online Resource eFig. 2). Although *N*1-methyladenosine was significantly greater in liver homogenate, it was not significantly different in kidney (eTable 2). No other differences were consistent across both liver and kidney compartments among candidate metabolites (e.g., *N*-2-methylguanosine, *N*-6-carbamoylthreonlyadenosine, or allantoin).

Discussion

In this outcome-dependent, stratified study of the GenIMS cohort, we found that the global metabolomic profile of plasma from CAP and sepsis subjects at ED presentation was broadly different among those who would subsequently live or die by 90 days. Using a non-targeted, mass-spectrometry approach, we uncovered small molecule differences consistent with known pathways abnormal in early sepsis. Of note, we showed that multiple nucleic acid metabolites were increased in non-survivors compared with survivors, and we confirmed these metabolites may act as DAMPs to potentiate the immune response in vitro.

Our data provide face validity to the measurement of small molecule metabolites as potential biomarkers in sepsis and pneumonia. More than prior data comparing disease with control, the metabolites we identified may associate with pathways leading to survival or death in sepsis. For example, primary and sulfated bile acids were elevated in plasma from non-survivors, consistent with mechanisms of inflammation-associated cholestasis. These include regulation of hepatocyte bile acid transporters at both the basolateral and canalicular membranes [25, 26], and structural and functional changes to hepatocyte tight junctions [27]. We also found typical changes in stress response pathways [28], with increased stimulation of the hypothalamic–pituitary–adrenal axis and elevation of plasma cortisol and corticosterone. We confirmed changes in protein catabolism observed in experimental sepsis [10, 13]. Visceral organs such as the liver require increased amino acids to synthesize proteins necessary for the immunologic response to infection, and our data may capture, in part, the interorgan transfer of amino acids after peripheral release [29]. Finally, allantoin, a marker of oxidative damage [30], was elevated in non-surviving sepsis and pneumonia patients. The enzyme catalyzing the oxidation of uric acid to allantoin is not found in humans [31], and this metabolite implicates greater relative activation of free radical oxidation.

We also found metabolites that may act as DAMPs when released from dying or damaged cells. The adaptive and innate immune response in sepsis is more than recognition of “non-self” or pathogen molecules, but augmented by “danger” molecules from abnormal host cell death, stress, or injury. These non-microbial DAMPs released from the cytosol of dying cells then activate both antigen-presenting cells and cytotoxic T-cells to potentiate the inflammatory cascade [32]. Known DAMPs including intracellular molecules such as uric

acid, adenosine metabolites, and double-stranded (ds)-DNA have endogenous adjuvant and pro-inflammatory activity, primarily by binding to molecular pattern receptors like TLR and NOD [33, 34]. Oxidative stress during sepsis may liberate these molecules allowing them to be detected by inflammatory effector cells [23]. We found that modified nucleosides, comprised primarily of RNA, were greater in the plasma of sepsis non-survivors compared to survivors. Such increase in nucleosides is also noted in serum of both multiple sclerosis and cancer [35, 36]. Although replication is required, our preliminary experiments identified metabolite candidates such as pseudouridine and allantoin that stimulated monocytes to release TNF α and/or IL1 β , similarly to known DAMPs like HMGB-1 [37].

When we used the global metabolomic profile to build prognostic models for patient survival from CAP sepsis at 90 days, classification of subjects was modest. We were unable to confirm the robust discrimination of metabolite-only models found in experimental studies of murine sepsis [10, 13]. Many factors, such as our small sample size, heterogeneity of sepsis course at the time of presentation, and the presence of exogenous metabolites, may account for differences in our clinical dataset compared to experimental models. As likely, though, is our choice to analyze plasma metabolomics, as prognostic performance may differ by choice of biofluid [10]. Future metabolomic prognostic models in sepsis will need rigorous assessment of their incremental value beyond less costly, more easily measured biomarkers, as well as comparison across organs.

For now, the variability in metabolic signatures that may occur in critical illness, cost of analyses, and complexity of data limit the routine study of small molecules. However, metabolomics may play a crucial role in discovery of disease pathways, particularly since genetic variability or variation in gene expression may not translate into actual functional differences [38, 39]. Important next steps in the metabolomics of pneumonia and sepsis are larger cohort studies comparing different sepsis phenotypes with healthy and non-infected controls, and targeted measurement of metabolite levels, while considering how metabolomics signatures may change over time and in response to treatment.

We recognize many limitations to our study. First, we used non-targeted mass spectrometry to determine relative levels of metabolites between cases and controls, and were unable to quantify metabolites. Although “targeted” or quantitative metabolomics is feasible with ¹H-NMR spectroscopy, this technique is less sensitive for low concentration molecules [40]. For our agnostic exploratory analysis, we preferred the most inclusive technique. Both the advantages and disadvantages of these methods are described elsewhere [9]. Second, we acknowledge that, without a validation cohort, the results here are hypothesis-generating. In a larger, independent sample, both novel metabolite pathways in sepsis and performance of metabolite-based prognostic models require confirmation. We were also unable to include many confounding factors in our unadjusted comparisons. These include the variability in pre-analytic time of samples drawn in the ED or administration of therapies (e.g., corticosteroids, dialysis). However, we successfully matched patients on pre-infection characteristics, while selecting extreme outcomes to uncover differences. A single metabolic snapshot cannot fully elucidate the mechanisms of small molecule disturbance. For example, the relative increase in urea in cases may result from reduced excretion by the injured kidney, ureagenesis in the liver, protein catabolism, or even exogenous intake [41]. Targeted metabolomics with relational databases to clinical parameters, protein expression, and putative enzymatic reactions are better suited to understanding the drivers of these complex pathways. Finally, we acknowledge that, although our monocyte stimulation experiments suggest specific nucleic acids act as putative DAMPs, these results require replication in independent cohorts and exploration of potential signaling pathways.

In summary, this preliminary study demonstrates that the global metabolomic profile is broadly different in subjects hospitalized with pneumonia and sepsis who died at 90 days compared to survivors. These data revealed physiologically relevant metabolites, consistent with known complex processes in early sepsis (bile acid metabolism, protein catabolism, inflammation, oxidative stress) that were different in survivors and non-survivors. Moreover, they demonstrate the potential for metabolomic signatures to uncover novel markers in sepsis, including putative DAMPs, though quantification and validation are necessary in larger studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by funding from the National Institutes of Health (K23 GM083215—Yende; 1K23GM104022-01-Seymour). The GenIMS study was funded by National Institute of General Medical Sciences and National Institutes of Health grant R01 GM61992 (Angus, Kellum) with additional support from GlaxoSmithKline for enrollment and clinical data collection and Diagnostic Products Corporation for the cytokine assays. These funding sources had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript. Bell and Mohny are employees of Metabolon, Inc. and, as such, have affiliations with or financial involvement with Metabolon, Inc.

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Fig. 1.

Heat map representation of unsupervised hierarchical clustering of 423 identified metabolites. *Shades of red or green* represent an increase or decrease, respectively, of the metabolite relative to the median metabolite levels of all patients. Controls are subjects who survived at 90 days, and cases are subjects who died on or before 90 days. Metabolites are grouped from *top to bottom* by metabolic pathways

Fig. 2. z-score plot for the metabolite data, normalized to the mean of control samples (truncated at 25 standard deviations for clarity). Controls are subjects who survived at 90 days, compared to subjects who died on or before 90 days. Metabolites are grouped from *top* to *bottom* by metabolic pathways

Fig. 3.

Schematic of nucleoside/nucleotide pathways using the Cytoscape platform (<http://www.cytoscape.org>). Hexagons represent metabolite compounds, with pathways simplified for illustration (a). Oxidative stress due to sepsis and pneumonia potentiates oxidation of xanthine to uric acid by xanthine oxidase (EC.1.17.3.2). Oxygen free radicals also oxidize urate/uric acid to allantoin/allantoate. In the current work, the relative amount of allantoin (b, *p* < 0.01) was greater in cases than controls. Oxidative stress may also induce RNA cleavage, releasing modified purine nucleosides (N₂, N₂ dimethylguanosine, pseudouridine) into the cytoplasm. We observed greater relative levels of plasma pseudouridine (c, *p* < 0.01, *q* < 0.1), N₂, N₂-dimethylguanosine (d, *p* < 0.01, *q* < 0.1), N₆-carbamoylthreonyladenosine (*p* < 0.05, *q* < 0.1, not shown), and N₁-methyladenosine (*p* < 0.05, *q* < 0.1, not shown) in cases. Horizontal bars correspond to group means, and dotted gray lines to matched pairs. After necrotic cell death, these nucleotide metabolites may further the inflammatory cascade by acting as damage-associated molecular patterns (DAMPs)

Fig. 4.

Metabolites stimulate monocytes/macrophages to induce cytokine production. TNF α (a) or IL1- β (b) level in supernatant from differentiated Thp1 monocytes stimulated with PBS (control), ultrapure LPS (1 ng/mL), recombinant human HMGB1 (2 μ g/mL), allantoin (0.3 mg/mL), 1-methyladenosine (5 μ M), N2-methylguanosine (5 μ M), N6-carbamoylthreonyladenosine (5 μ M) or beta-pseudouridine (10 mM) for 16 h. TNF α level (c) in supernatants from differentiated Thp-1 monocytes treated with PBS (control), LPS (4 ng/mL), HMGB1 (5 μ g/mL) or beta-pseudouridine (0.0001–10 mM) for 16 h. TNF α level (d) in supernatants from differentiated Thp-1 monocytes treated with PBS (control), LPS (4 ng/mL), HMGB1 (5 μ g/mL) or beta-pseudouridine (10 mM) alone or in combination with polymyxin B (PMB; 10 μ M) or VIPER (TLR4-antagonist; 10 μ L) for 16 h. Data represent mean $\pm$ SEM. * p < 0.05 vs. control; # p < 0.05 vs. alone. Data representative of three separate experiments performed in duplicate or triplicate

Table 1
Characteristics of cases and controls

Characteristic	All (<i>n</i> = 30)	Cases (<i>n</i> = 15)	Controls (<i>n</i> = 15)	<i>p</i> value ^a
Matching characteristics				
Age, years, median (IQR)	79 (75–82)	79 (76–82)	78 (73–83)	0.95
Male sex, <i>n</i> (%)	16 (53)	8 (53)	8 (53)	0.99
White race, <i>n</i> (%)	30 (100)	15 (100)	15 (100)	0.99
Microbiology, <i>n</i> (%) ^b				
Gram positive	16 (53)	9 (60)	7 (47)	0.53
Gram negative	5 (17)	2 (13)	3 (20)	0.65
Procalcitonin >0.25 mg/mL, <i>n</i> (%)	17 (57)	9 (60)	8 (53)	0.65
Disease severity and comorbidity				
Ever smoked, <i>n</i> (%)	19 (63)	8 (53)	11 (73)	0.26
Charlson co-morbidity index, median (IQR)	2 (1–3)	3 (1–4)	1 (1, 2)	0.02
Charlson greater than zero, <i>n</i> (%)	26 (87)	14 (93)	12 (80)	0.16
Enrollment PSI, median (IQR)	107 (76–139)	125 (105–142)	82 (0–120)	0.02
APACHE III score, median (IQR)	75 (57–80)	76 (74–95)	59 (53–75)	0.02
Enrollment biomarker levels, median (IQR)				
Interleukin-6, pg/mL	196 (42–1,662)	433 (196–2,663)	65 (11–443)	0.39
Interleukin-10, pg/mL	32.8 (8.6–65.1)	32.8 (12.3–64)	43.5 (7.3–216)	0.15
Tumor necrosis factor, pg/mL	11.6 (8.8–22.8)	12.3 (10.2–24.8)	10.4 (7.8–18.6)	0.96
Outcomes				
Antibiotics within 8 h of presentation, <i>n</i> (%)	29 (97)	15 (100)	14 (93)	0.32
Organ failure during hospitalization, <i>n</i> (%)	28 (93)	12 (80)	2 (13)	<0.01
Hospital length of stay, days, median (IQR)	9 (6–14)	11 (6–15)	7 (5–11)	0.16
Intensive care use, <i>n</i> (%)	11 (37)	8 (53)	3 (20)	0.06
Mechanical ventilation use, <i>n</i> (%)	8 (27)	8 (53)	0 (0)	<0.01

APACHE III Acute Physiology and Chronic Health Evaluation III, IQR interquartile range, PSI Pneumonia severity index

^a Comparing cases and controls, using Wilcoxon signed rank test or paired Student's *t* test, as appropriate. Proportions were compared using Chi square or Fisher's exact test, as appropriate

^b Among patients in whom gram stain of organism was known (*n* = 21, 70 %)

Examples of significant metabolites comparing non-survivors (cases) and survivors (controls) of pneumonia and sepsis at 90 days

Table 2

Super pathway	Sub pathway	Metabolite	Relative abundance ^a		Exact <i>p</i> value ^b
			Cases	Controls	
Amino acids	Serine	<i>N</i> -acetyl serine	1.23 (1.01–2.32)	0.79 (0.61–1.00)	0.007
	Tryptophan	<i>C</i> -glycosyltryptophan	1.67 (0.77–2.57)	0.89 (0.63–1.09)	0.007
		Kynurenate	1.30 (0.88–4.29)	0.64 (0.41–0.97)	0.007
Carbo-hydrate	Urea cycle	Urea	1.33 (0.97–1.78)	0.62 (0.53–1.02)	0.008
	Erythro sugars metabolism	Erythronate	1.82 (0.94–3.83)	0.94 (0.69–1.10)	0.008
	Mannose metabolism	Mannitol	1.84 (1.00–3.32)	0.68 (0.25–1.00)	0.007
	Glycolysis	Glycerate	1.18 (0.95–1.81)	0.81 (0.57–1.04)	0.007
	Nucleotide sugar	Xylionate	2.24 (0.85–2.99)	0.84 (0.64–1.07)	0.004
Energy	Krebs cycle	Fumarate	1.19 (0.95–1.68)	0.85 (0.61–1.02)	0.005
Lipids	Bile acid metabolism	Glycochenolate sulfate	1.64 (1.09–2.09)	0.79 (0.55–0.95)	0.001
		Taurochenolate sulfate	1.72 (1.38–4.48)	0.60 (0.47–0.88)	<0.001
	Long chain fatty acid	10-heptadecenoate (17:1n7)	1.13 (0.92–1.92)	0.78 (0.52–1.45)	0.008
	Lysolipid	2-Oleoylglycero-phosphoethanolamine	0.43 (0.43–0.72)	1.20 (0.99–1.46)	0.005
		1-Linoleoylglycero-phosphoethanolamine	0.73 (0.46–1.05)	1.75 (0.93–2.52)	0.007
	Steroid metabolism	Cortisol	1.55 (0.94–2.35)	0.50 (0.30–1.08)	0.004
	Purine/adenosine metabolism	<i>N</i> 1-methyladenosine	1.19 (0.97–1.31)	0.96 (0.88–1.00)	0.002
	Pyrimidine metabolism	Pseudouridine	1.32 (0.95–2.26)	0.87 (0.75–1.07)	0.003
	Tocopherol metabolism	Alpha-CEHC glucuronide	0.82 (0.40–2.85)	0.36 (0.36–0.36)	0.002
	Vitamin B6	Pyridoxate	3.96 (1.07–10.96)	0.63 (0.49–0.96)	<0.001
Cofactors/vitamins	Nicotinate and nicotinamide metabolism	<i>N</i> 1-methyl-2-pyridone-5-carboxamide	1.98 (0.99–3.67)	0.71 (0.53–1.02)	0.008
	Xanthine metabolism	Paraxanthine	0.35 (0.14–0.90)	1.17 (0.68–1.67)	0.004
		Caffeine	0.34 (0.15–1.09)	1.14 (0.69–2.24)	0.010
Xenobiotics	Sugar substitute	Erythritol	1.71 (0.96–3.20)	0.87 (0.62–1.08)	0.008

Metabolites selected for illustration if *p* value 0.01 and *q* value <0.1

^aRelative abundance determined as median (IQR) of normalized, imputed raw area counts

^b*p* values determined using the Wilcoxon signed rank test