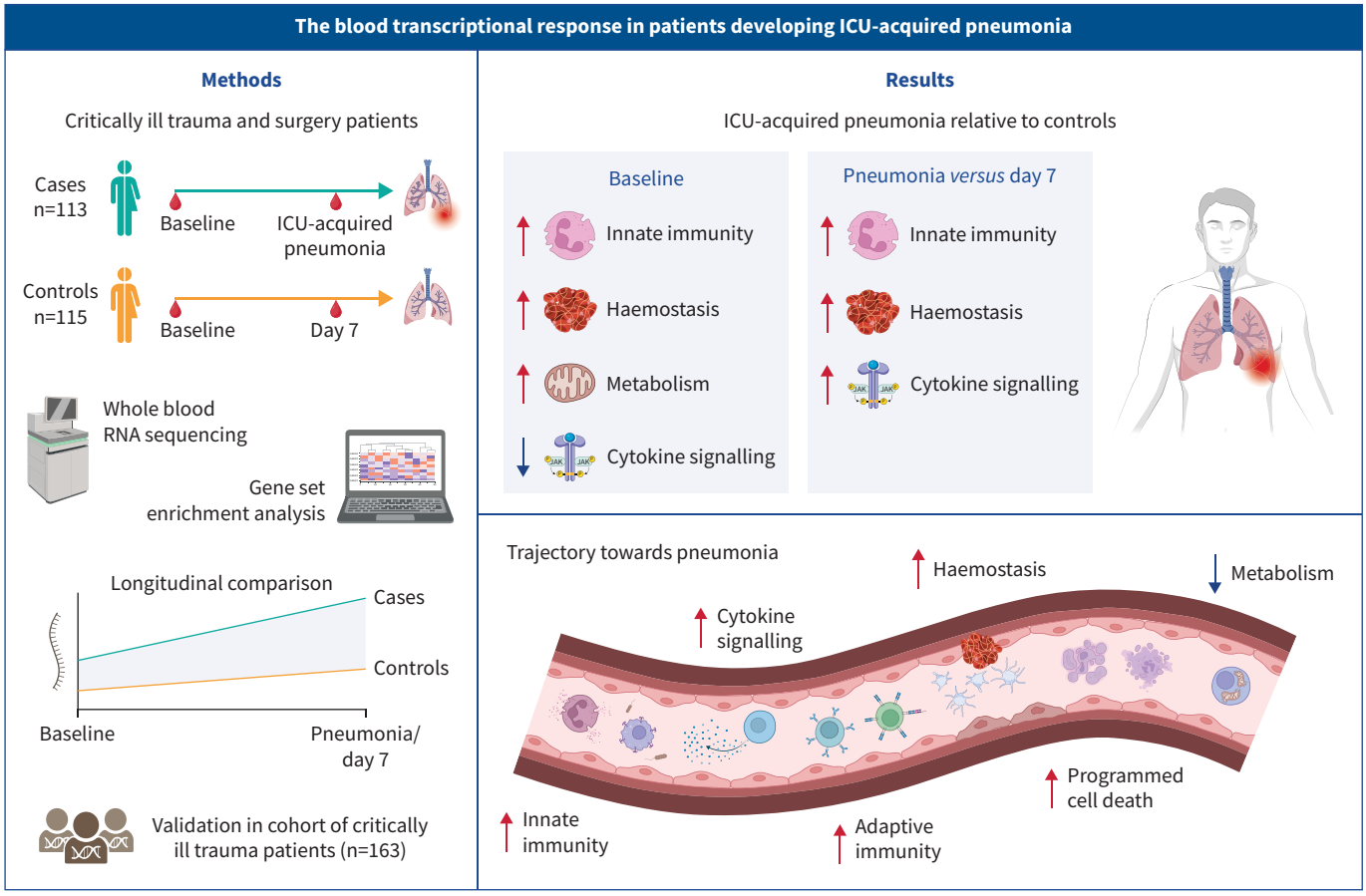
The blood transcriptional response in patients developing intensive care unit-acquired pneumonia

Justin de Brabander, Erik H.A. Michels, Joe M. Butler, Tom D.Y. Reijnders, Tjitske S.R. van Engelen, Giuseppe G.F. Leite, Fleur P. Paling, Augustijn M. Klarenbeek, Daoud L.S. Sie, Roxane E. Boyer, Timothy E. Sweeney, Marc J.M. Bonten, Leen Timbermont, Surbhi Malhotra-Kumar, Jan A.J.W. Kluytmans, Hessel Peters-Sengers and Tom van der Poll on behalf of the ASPIRE-ICU study team



GRAPHICAL ABSTRACT Overview of the study. ICU: intensive care unit. Partially created using BioRender.com.

The blood transcriptional response in patients developing intensive care unit-acquired pneumonia

Justin de Brabander [1], Erik H.A. Michels [1], Joe M. Butler [1], Tom D.Y. Reijnders [1], Tjitske S.R. van Engelen [1], Giuseppe G.F. Leite [1,2], Fleur P. Paling [3], Augustijn M. Klarenbeek [1], Daoud L.S. Sie [4], Roxane E. Boyer [4], Timothy E. Sweeney [5], Marc J.M. Bonten [3], Leen Timbermont [6], Surbhi Malhotra-Kumar [6], Jan A.J.W. Kluytmans [7], Hessel Peters-Sengers [1,8] and Tom van der Poll [1,9] on behalf of the ASPIRE-ICU study team

[1]Center for Experimental and Molecular Medicine (CEMM), Amsterdam UMC location University of Amsterdam, Amsterdam, The Netherlands. [2]Division of Infectious Diseases, Department of Medicine, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil. [3]Julius Center for Health Sciences and Primary Care, UMC Utrecht, Utrecht University, Utrecht, The Netherlands. [4]Department of Human Genetics, Amsterdam UMC location Vrije Universiteit, Amsterdam, The Netherlands. [5]Inflammatix Inc., Sunnyvale, CA, USA. [6]Laboratory of Medical Microbiology, Vaccine and Infectious Disease Institute, University of Antwerp, Antwerp, Belgium. [7]Department of Medical Microbiology, UMC Utrecht, Utrecht University, Utrecht, The Netherlands. [8]Department of Epidemiology and Data Science, Amsterdam UMC location Vrije Universiteit, Amsterdam, The Netherlands. [9]Division of Infectious Diseases, Amsterdam UMC location University of Amsterdam, Amsterdam, The Netherlands.

Corresponding author: Justin de Brabander (j.debrabander@amsterdamumc.nl)

Shareable abstract (@ERSpublications)
Patients developing ICU-acquired pneumonia, relative to ICU controls, exhibit distinct changes in blood transcriptomes from baseline to pneumonia diagnosis, indicating broad immune dysfunction across multiple host response pathways https://bit.ly/4gRl8E2

Cite this article as: de Brabander J, Michels EHA, Butler JM, *et al*. The blood transcriptional response in patients developing intensive care unit-acquired pneumonia. *Eur Respir J* 2025; 65: 2400592 [DOI: 10.1183/13993003.00592-2024].

This article has an editorial commentary: https://doi.org/10.1183/13993003.00185-2025

Received: 25 March 2024
Accepted: 23 Dec 2024

Abstract

Background Immune response dysregulation has been implicated in the development of intensive care unit (ICU)-acquired pneumonia. We aimed to determine differences in the longitudinal blood transcriptional response between patients who develop ICU-acquired pneumonia (cases) and those who do not (controls).

Methods We performed a case–cohort study in mechanically ventilated trauma and surgery patients with ICU stays >2 days, enrolled in 30 hospitals across Europe. We collected blood for RNA sequencing at baseline, day 7 and (in cases) the day of pneumonia diagnosis. We performed gene set enrichment analysis and analysed longitudinal gene expression changes using linear mixed models. External validation was performed using an independent trauma cohort.

Results We enrolled 113 cases and 115 controls, with similar baseline characteristics. At baseline (median 2 days after ICU admission), cases showed upregulated gene pathways relating to innate immunity, haemostasis and metabolism, and downregulated adaptive immune pathways. These changes persisted at the day of pneumonia diagnosis (median 6 days, compared to day 7 in controls). In the longitudinal comparison, cases exhibited enhanced upregulation of innate immunity, adaptive immunity and haemostasis pathways, along with enhanced downregulation of metabolism pathways, relative to controls (all $p<0.00001$, except haemostasis $p<0.05$). These findings were largely externally validated. Cases had higher quantitative sepsis response signature scores ($p<0.001$), reflective of immune dysregulation.

Conclusion Patients developing ICU-acquired pneumonia exhibit distinct blood transcriptional responses shortly after ICU admission and in the subsequent path to pneumonia, suggestive of broad immune dysfunction with both immunosuppressive and inflammatory features.

Introduction

Intensive care unit (ICU)-acquired pneumonia is a common complication in critically ill patients [1, 2], with risk factors including mechanical ventilation and colonisation with pathogenic bacteria such as *Staphylococcus aureus* [3, 4]. Recent literature has highlighted critical illness-associated immunosuppression

as a key contributor to ICU-acquired infections, leading to several clinical trials testing immunostimulants to prevent new ICU infections, including pneumonia [5–9].

Besides immunosuppression, critically ill patients portray broad host response disturbances, including systemic inflammation, endothelial activation and pro-coagulant responses [6, 7, 10]. Our group demonstrated that sepsis patients with secondary ICU-acquired infections, compared to those without, displayed wide-ranging host response aberrations across multiple pathophysiological domains [11]. In agreement, we showed that patients developing ICU-acquired pneumonia exhibited a more dysregulated host response, characterised by hyperinflammation and endothelial barrier dysfunction, from baseline to pneumonia diagnosis [12].

Blood transcriptome analyses have highlighted immune dysregulation, encompassing both immune activation and suppression, in critically ill patients [10, 13–17]. Our group found no differences in blood transcriptome profiles at ICU admission between sepsis patients with and without secondary infection [18]. Currently, longitudinal analyses of blood leukocyte mRNA profiles from admission to ICU-acquired infection diagnosis are lacking. Here, we aimed to determine differences in the longitudinal blood transcriptional response between critically ill patients who did or did not develop ICU-acquired pneumonia. Analyses were performed shortly after ICU admission and at pneumonia diagnosis or after a comparable ICU stay for those without pneumonia. We hypothesised that patients with ICU-acquired pneumonia, compared to those without, show broad alterations in immune pathways, involving both pro-inflammatory and immunosuppressive changes.

Methods

Study population and design

Patients were from the ASPIRE-ICU (Advanced understanding of *Staphylococcus aureus* and *Pseudomonas aeruginosa* Infections in EuRopE – Intensive Care Units) project, a prospective study across 30 hospitals in 11 European countries. Patient selection was described elsewhere [19]. In short, mechanically ventilated patients with an expected ICU stay ⩾2 days, based on clinical judgement, were enrolled within 3 days of ICU admission. Inclusion followed a 1:1 ratio of *S. aureus*-colonised and non-colonised patients. The presence of pneumonia was assessed daily using the Centers for Disease Control and Prevention definitions [20], involving radiographic infiltrates, respiratory deterioration, systemic infection signs and consistent physical findings. Details are provided in the supplementary methods [19]. Recruitment occurred from June 2015 to October 2018. The study protocol was approved by the institutional review boards or ethical committees in each country and/or site. All participants or their legally authorised representative provided written informed consent.

We selected patients with trauma or surgery as primary ICU admission reason to minimise heterogeneity and determine the onset of immunological dysfunction. Cases were patients who developed protocol-defined ICU-acquired pneumonia >2 and ⩽14 days after ICU admission. Controls were patients without pneumonia during ICU admission, who were in the ICU >2 days. Controls were randomly selected in a 1:1 ratio to cases, adhering to the “study base principle”, ensuring both groups originate from the same base population (*i.e.* critically ill trauma and surgery patients with ICU stays >2 days). This approach, avoiding matching and its potential uncorrectable biases, allows for effective confounder adjustment in subsequent statistical analysis [21]. Longitudinal transcriptomic data were validated in an independent ICU cohort of trauma patients (see supplementary material for details) [13].

Blood was collected in PAXgene tubes (Qiagen, Venlo, The Netherlands) upon study enrolment (“baseline”), on the day of pneumonia diagnosis (“event”) in cases and on day 7 after enrolment in controls. RNA sequencing was performed as described in the supplementary methods. Gene expression data are accessible under NCBI BioProject ID PRJNA1008068.

Statistical analysis

For details, see supplementary material. We used DESeq2 to analyse gene expression differences between cases and controls *via* moderated Wald tests [22]. For gene set enrichment analysis, we used Reactome-defined immune-related pathways [23]. We compared cases and controls at two time-points: baseline and day of event for cases *versus* day 7 for controls. As an unsupervised approach, baseline gene coexpression network analysis was performed using CEMiTool [24].

We assessed the longitudinal transcriptional response from baseline to event (for cases) or day 7 (for controls) by applying linear mixed models to each gene, estimating their individual change over time (glmmSeq) [25]. Fixed effects included study group, ICU sampling day and their interaction. From the

model output, we calculated gene-specific z-scores of the interaction term, indicating gene expression changes in cases relative to controls. These z-scores were used in gene set enrichment analysis, identifying pathways with more pronounced changes over time. In additional analyses, we adjusted for predefined potential confounders: study location, Acute Physiology and Chronic Health Evaluation (APACHE) score (containing age and immunosuppressed state), sex, ICU admission reason (trauma, elective or emergency surgery), *S. aureus* colonisation, mechanical ventilation, sampling time and (in the validation cohort) high-dose steroids treatment (supplementary figure S1).

Results

Patient characteristics

ASPIRE-ICU enrolled 873 patients following ICU admission after trauma or surgery (supplementary figure S2). Among 854 patients in the ICU >2 days, 117 (13.7%) developed ICU-acquired pneumonia >2 and ⩽14 days post-admission (cases), while 715 (83.7%) never developed pneumonia (controls). 22 patients diagnosed with pneumonia outside this time frame were excluded. We randomly selected a subgroup of controls (1:1 ratio to cases, n=117); two controls without available samples were excluded. Selected (n=115) and non-selected (n=598) controls had similar characteristics (supplementary table S1). Four cases diagnosed with pneumonia on enrolment day were excluded, since their baseline and event sampling coincided. Baseline characteristics were comparable between cases and controls (table 1 and supplementary tables S2 and S3). Cases had longer ICU stays, but mortality was similar. The median (interquartile range (IQR)) time to ICU-acquired pneumonia was 6 (4–8) days (supplementary figure S3). Among the 113 cases, 92 (81.4%) had ventilator-associated pneumonia. The causative pathogen was *S. aureus* in 29 cases (25.7%) and *Pseudomonas aeruginosa* in 17 cases (15.0%). Respiratory pathogens were similar between colonised and non-colonised cases, expect for *S. aureus*, which was more frequent in colonised cases (supplementary table S4).

Blood leukocyte transcriptome analysis

We compared blood transcriptomes between cases and controls at baseline (median (IQR) 2 (1–3) days after ICU admission) and at the day of pneumonia diagnosis (in cases) *versus* day 7 after study enrolment (in controls). Cases had 112 baseline samples (99.1%) and 106 event samples (93.8%) available (supplementary figure S4). Among controls, we obtained 114 baseline samples (99.1%) and 65 samples at day 7 (56.5% of all controls, 87.8% of those still in the ICU). Of the 41 controls no longer in the ICU on day 7, 32 had been discharged and nine had died. After mRNA pre-processing, 23 105 genes were included. At baseline, 1076 (4.7%) genes were differentially expressed (figure 1a and supplementary table S5). Pathway enrichment analysis demonstrated that cases exhibited upregulation in innate immune, haemostasis and metabolism pathways, and downregulation in adaptive immune and cytokine signalling pathways (figure 1b and supplementary table S6). At the time of event (for cases) *versus* day 7 (for controls), 491 (2.4%) genes were differentially expressed (figure 1a). Pathway analysis yielded similar results to the baseline comparison, except for upregulation of the cytokine signalling pathway in cases during ICU-acquired pneumonia (*versus* downregulation at baseline) (figure 1b and supplementary table S7). Our findings remained consistent after accounting for potential confounders (supplementary figure S5).

Event samples were obtained between day 3 and 14 after ICU admission (supplementary figure S3). Since control follow-up samples were taken on day 7 (median (IQR) 8 (8–8) days post-admission), we performed a sensitivity analysis only using event samples from days 6–10 (n=41) to align time-points. This yielded similar results to the primary analysis (supplementary figure S6). Further analysis, limited to cases and controls with two samples, showed comparable outcomes (supplementary table S8 and supplementary figure S7). A baseline comparison exclusively in patients with ICU stays ⩾7 days, seeking to minimise informative loss of controls, revealed fewer differentially expressed genes but consistent gene set enrichment results (supplementary table S9 and supplementary figure S8).

To obtain further insight into baseline biological mechanisms associated with ICU-acquired pneumonia development, we performed a gene coexpression analysis using baseline transcriptomic data. 12 modules of coexpressed genes were identified, each representing a cluster of correlated genes likely involved in similar molecular functions. Four modules differed significantly between cases and controls (supplementary table S10). Among these, only two, both downregulated in cases, were significantly enriched for Reactome pathways (figure 2a). Module 12 involved genes in interferon and cytokine signalling, while Module 2 included genes related to lymphocyte function and adaptive immunity, such as T-cell receptor signalling and CD28 costimulation (figure 2b and c). Thus, this unsupervised approach corroborated the results shown in figure 1b (*i.e.* downregulated cytokine signalling and adaptive immune pathways at baseline in cases).

TABLE 1 Patient characteristics and clinical outcomes

	Cases (n=113)	Controls (n=115)	p-value
Demographics			
Age, years	66 (53–75)	61 (49–71)	0.07
Male	85 (75.2)	80 (69.6)	0.42
Body mass index, kg·m^{-2}	26.2 (23.7–29.4)	26.0 (23.1–29.2)	0.70
Charlson Comorbidity Index[#]	3 (1–5)	3 (1–5)	0.76
Clinical characteristics at ICU admission			
ICU admission reason[¶]			0.93
Elective surgery	11 (9.7)	12 (10.4)	
Emergency surgery	49 (43.4)	52 (45.2)	
Trauma	53 (46.9)	51 (44.3)	
Colonised with *Staphylococcus aureus*	62 (54.9)	51 (44.3)	0.15
Heart rate, beats·min^{-1}	100 (67–120)	104 (80–120)	0.70
Mean arterial pressure, mmHg	76 (60–105)	73 (63–101)	0.71
Temperature, °C	36.9 (36.0–37.8)	37.0 (35.8–37.8)	0.66
Oxygen saturation, %	96 (93–99)	97 (94–99)	0.29
APACHE II score	18 (13–22)	18 (13–24)	0.47
APACHE IV score	64 (41–93)	55 (31–90)	0.23
Laboratory values			
White blood cells, ×10^{9} L^{-1}	12.9 (8.3–17.1)	13.7 (9.2–17.7)	0.40
Neutrophils, ×10^{9} L^{-1+}	11.4 (7.0–14.4)	11.4 (7.8–15.9)	0.43
Lymphocytes, ×10^{9} L^{-1+}	0.8 (0.5–1.2)	0.8 (0.5–1.1)	0.85
Platelets, ×10^{9} L^{-1}	191 (133–251)	191 (144–275)	0.36
C-reactive protein, mg·dL^{-1+}	39 (6–131)	29 (5–155)	0.74
Antibiotics use in 2 weeks prior to admission[+]	24 (24.2)	29 (29.6)	0.49
Longitudinal characteristics			
SOFA score at day 4	6 (4–8)	4 (2–7)	0.004
SOFA score at day 7	5 (3–8)	4 (3–7)	0.09
White blood cells at day 4, ×10^{9} L^{-1}	11.1 (8.4–16.0)	10.9 (8.2–14.4)	0.44
White blood cells at day 7, ×10^{9} L^{-1}	14.2 (10.7–19.1)	12.7 (9.9–15.5)	0.06
Platelets at day 4, ×10^{9} L^{-1}	186 (129–270)	205 (146–263)	0.50
Platelets at day 7, ×10^{9} L^{-1}	279 (178–387)	272 (192–357)	0.79
ICU-acquired pneumonia characteristics			
Onset of ICU-acquired pneumonia, days	6 (4–8)		
SOFA score	6 (4–9)		
Ventilator-associated pneumonia	92 (81.4)		
Pathogen cultured from respiratory sample			
Acinetobacter spp.	22 (19.5)		
Candida spp.	9 (8.0)		
Klebsiella pneumoniae	9 (8.0)		
Proteus mirabilis	6 (5.3)		
Pseudomonas aeruginosa	17 (15.0)		
Staphylococcus aureus	29 (25.7)		
Other[§]	20 (17.7)		
Adequate antibiotic treatment initiated	104 (92.0)		
Clinical outcomes			
Length of ICU stay, days	15 (11–30)	9 (5–15)	<0.001
Length of mechanical ventilation, days	13 (8–21)	6 (3–11)	<0.001
Readmission <30 days of ICU discharge[+]	6 (7.4)	3 (3.3)	0.48
ICU mortality	32 (26.9)	23 (21.1)	0.20
30-day mortality	25 (23.1)	31 (29.5)	0.37
90-day mortality	46 (44.2)	40 (38.5)	0.48

Data are presented as median (interquartile range) or n (%), unless otherwise stated. ICU: intensive care unit; APACHE: Acute Physiology and Chronic Health Evaluation; SOFA: Sequential Organ Failure Assessment. [#]: a comprehensive list of individual comorbidities is detailed in supplementary table S2; [¶]: for detailed breakdowns of ICU admission reasons, see supplementary table S3; [+]: these variables had >10% missing data (Chi-squared tests confirmed this missingness to be "at random"; see supplementary methods for details); [§]: other cultured pathogens included *Enterobacter cloacae* (n=5), *Escherichia coli* (n=4), *Haemophilus influenzae* (n=3), *Providencia* spp., *Aspergillus fumigatus* (n=2 each), *Citrobacter koseri*, coagulase-negative staphylococci, *Corynebacterium* spp. and *Raoultella planticola* (n=1 each) (note: pathogens reflect culture results of respiratory specimens, which do not necessarily assign these as causative for pneumonia).

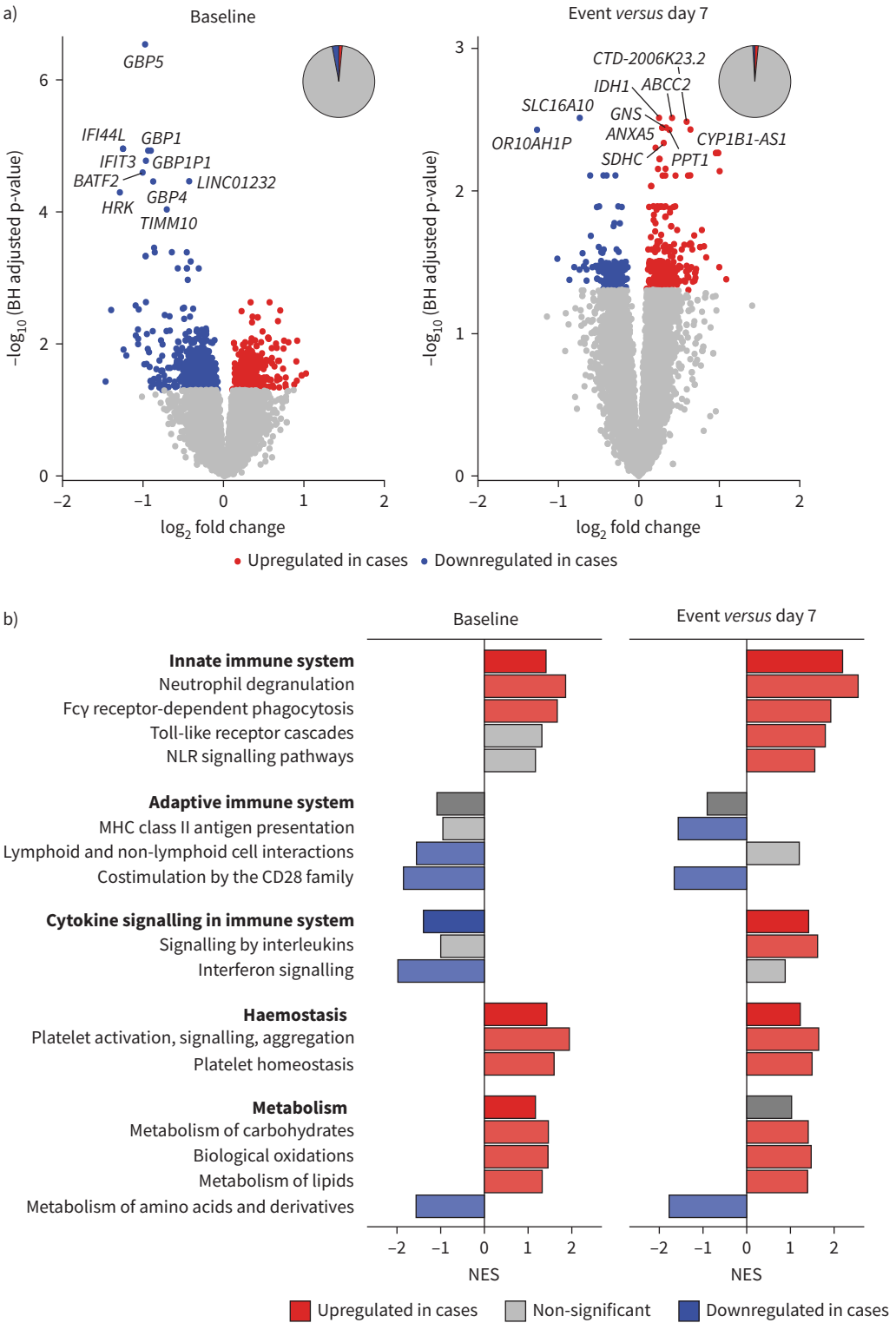


FIGURE 1 Blood transcriptomes in critically ill trauma and surgery patients who did or did not develop intensive care unit (ICU)-acquired pneumonia. a) Volcano plots illustrating the differences in leukocyte genomic responses (integrating $\log_2$ fold changes and Benjamini–Hochberg (BH) adjusted probabilities) between patients who did or did not develop ICU-acquired pneumonia (cases and controls, respectively), at baseline (112 cases and 114 controls) and at the time of pneumonia diagnosis in cases (n=106) *versus* at day 7 for controls (n=65). The top 10 differentially expressed genes are labelled. Blue dots represent significantly underexpressed genes in cases (*versus* controls), whereas red dots represent significantly overexpressed genes

in cases (*versus* controls). Within plots, pie charts show the extent of gene expression changes: blue slices show significantly underexpressed genes, red slices show significantly overexpressed genes and grey slices show the proportion of genes not different between groups. Depicted gene names are detailed in supplementary table S5. b) Reactome pathway analysis. The magnitude of expression is portrayed using the normalised enrichment score (NES), which indicates overrepresentation (positive NES) or underrepresentation (negative NES) of pathways in cases compared to controls. The analysis was performed on the following parent pathways: innate immune system, adaptive immune system, cytokine signalling in immune system, programmed cell death, haemostasis and metabolism. Only parent pathways and those that were significant in one of the comparisons are shown. Grey bars represent non-significantly different expressed pathways. The parent pathways, indicated in bold, are followed by their contributing child pathways. See supplementary tables S6 and S7 for pathway descriptions and Reactome IDs.

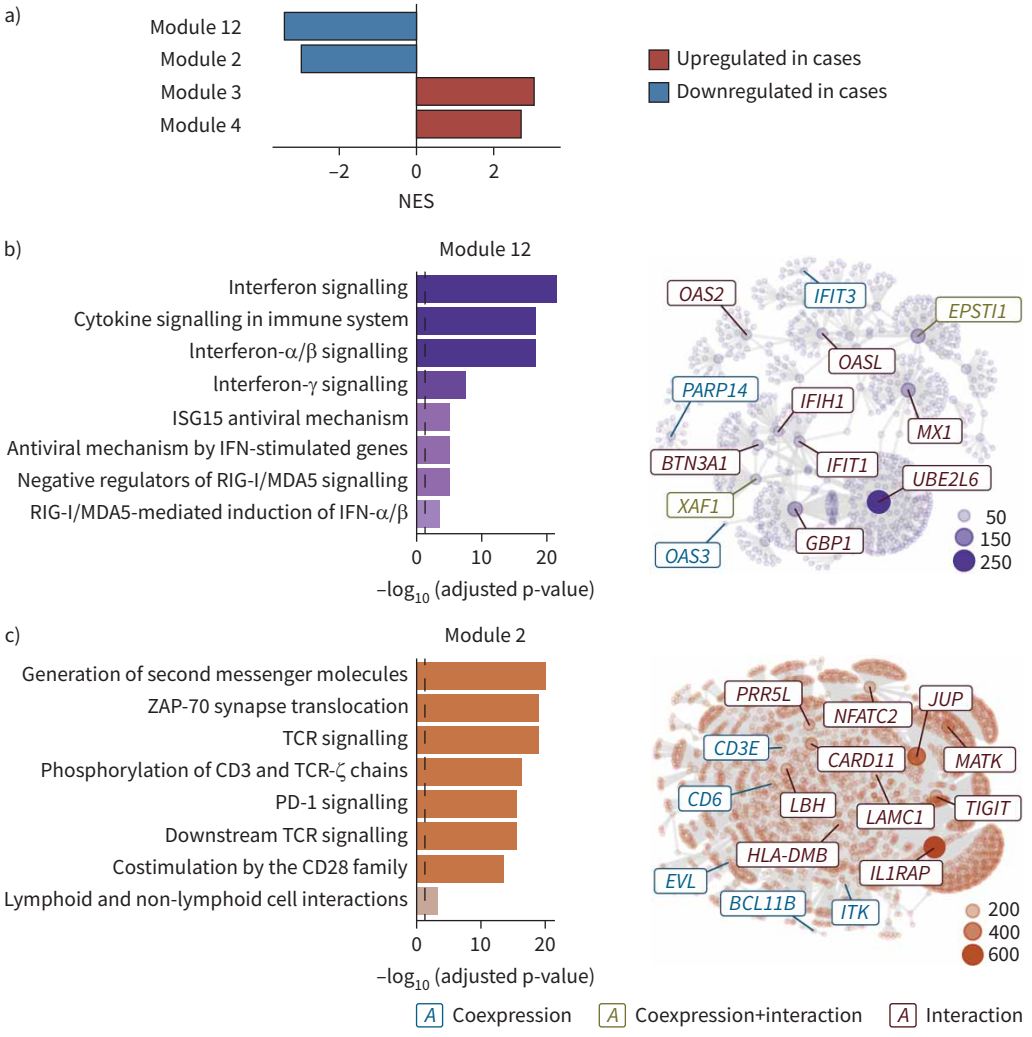


FIGURE 2 Gene coexpression analysis in critically ill trauma and surgery patients who did *versus* did not develop intensive care unit-acquired pneumonia, performed using baseline transcriptomes. a) Gene set enrichment analysis of four coexpressed gene modules in cases *versus* controls. The magnitude of expression is portrayed using the normalised enrichment score (NES), indicating overrepresentation or underrepresentation of the gene module in cases *versus* controls. b, c) Top eight over-represented Reactome pathways in b) Module 12 and c) Module 2, and protein–protein interaction (PPI) and correlation networks of genes in each module. Each dot (hub) represents a gene and lines connecting the genes indicate gene correlations or PPIs (illustrated by hub colours). The hub size reflects the degree of connectivity (*i.e.* the number of connections each gene has with other genes in the network).

Taken together, these data indicate that patients developing ICU-acquired pneumonia show altered gene expression profiles in blood leukocytes prior to and at the time of pneumonia diagnosis across multiple immune-related pathways, suggestive of both enhanced inflammation (innate immunity and haemostasis) and immune suppression (adaptive immunity).

Longitudinal blood transcriptional response

To obtain insight into the progression of the blood transcriptional response from baseline to pneumonia diagnosis, we longitudinally compared cases and controls using linear mixed models. Over time, cases demonstrated enhanced upregulation in pathways related to innate and adaptive immunity, cytokine signalling, programmed cell death (all $p<0.00001$) and haemostasis ($p<0.05$), indicating a relative increase in genes within these pathways compared to controls (figure 3 and supplementary table S11). Concurrently, cases showed progressive downregulation of metabolism-related pathways relative to controls ($p<0.00001$). We validated these findings in an independent US cohort of critically ill trauma patients with longitudinal sampling, consisting of 45 cases and 118 controls (supplementary table S12) [13]. In this

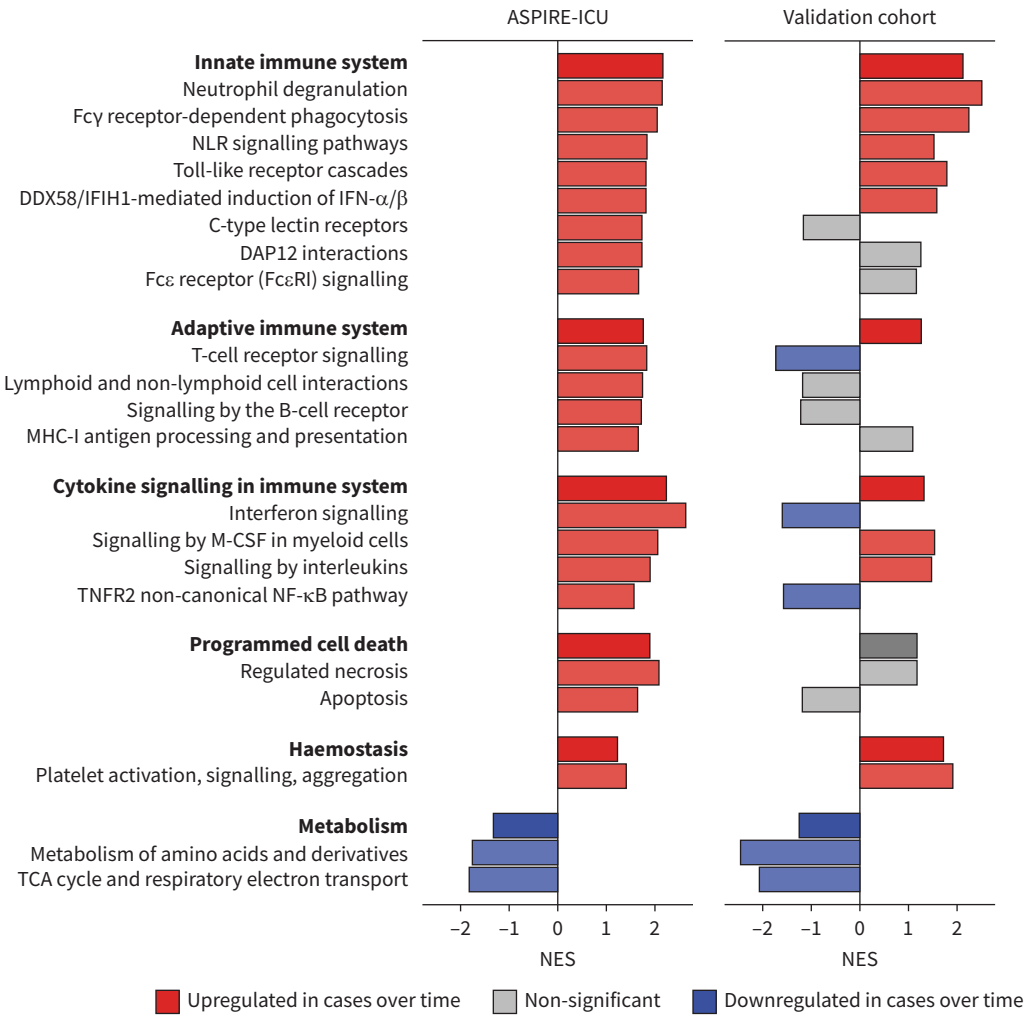


FIGURE 3 Pathway analysis of the longitudinal transcriptional response in patients who developed intensive care unit-acquired pneumonia, relative to patients who did not, as assessed by linear mixed models. The analysis used samples obtained at two time-points (baseline and time of pneumonia diagnosis in cases or day 7 in controls) from the ASPIRE-ICU cohort (113 cases and 115 controls) and the validation cohort (45 cases and 118 controls). The normalised enrichment score (NES) represents the magnitude of pathway expression changes in cases relative to controls over time, based on interaction terms from linear mixed models. Only pathways that were significant in the derivation cohort are shown. Parent pathways, indicated in bold, are followed by their contributing child pathways. See supplementary tables S6 and S7 for pathway descriptions and Reactome IDs.

cohort, baseline characteristics were similar between groups, except for more males and higher APACHE II scores in cases. In the unadjusted analysis, this independent cohort confirmed all the main ("parent") Reactome pathway differences observed in the derivation cohort, except for upregulation of the programmed cell death pathway (figure 3). For the branching ("child") pathways, some differences between cohorts were present, particularly in the adaptive immunity pathway (as indicated by asterisks in supplementary table S11). Adjusting for potential confounders yielded comparable findings (supplementary figure S9). Re-analysis of the derivation cohort after stratification by trauma or surgery admission reason revealed no major differences in gene pathway trajectories (supplementary figure S10). Supplementary figure S11 depicts each pathway's core enrichment genes.

In the derivation cohort, 25 pneumonia cases diagnosed after day 7 had two pre-diagnosis samples collected (baseline and day 7). In the validation cohort, 30 additional samples from cases (20 on day 4 and 10 on day 7) and 79 from controls (all on day 4) were available. Including these additional samples largely replicated our gene set enrichment findings, except for the haemostasis (derivation) and adaptive immunity (validation) parent pathways (supplementary figure S12). A sensitivity analysis exclusively in patients with ICU stays $\geqslant$7 days, aimed at minimising informative dropout among controls, also showed consistent results (supplementary table S9 and supplementary figure S13).

Collectively, these data indicate that critically ill patients who develop ICU-acquired pneumonia, relative to patients who do not, show significantly different gene expression trends in blood leukocytes from baseline to event, relating to relatively enhanced innate immune activation and reduced cellular metabolism.

Pneumonia caused by S. aureus versus P. aeruginosa

We compared patients with ICU-acquired pneumonia caused by *S. aureus* (n=27) or *P. aeruginosa* (n=15) (supplementary table S13). At baseline, *S. aureus* pneumonia, relative to *P. aeruginosa*, was associated with reduced expression in pathways related to innate immunity, programmed cell death, haemostasis and metabolism (supplementary figure S14a). Longitudinally, *S. aureus* pneumonia patients exhibited increasing expression of innate immunity and metabolism pathways over time, relative to *P. aeruginosa* (supplementary figure S14b).

Molecular endotypes

We used hierarchical clustering to investigate the transcriptomic response of different baseline molecular endotypes. This analysis identified two distinct molecular endotypes (supplementary table S14 and supplementary figure S15a), each exhibiting features of both inflammation and immunosuppression (supplementary figure S15b).

RNA signatures

We next evaluated established RNA signatures of immune dysregulation (figure 4). The quantitative sepsis response signature (SRSq) score (range 0–1) reflects immune dysregulation severity [17]. The monocyte state 1 (MS1) cell fraction, estimated from gene expression, indicates the abundance of MS1 cells in whole blood [16]. MS1 cells resemble myeloid-derived suppressor cells and exert immunosuppressive features [16]. In our cohort, SRSq scores were higher in cases at both baseline and event, with similar decreases over time in both groups ($p<0.001$) (figure 4a). MS1 scores were higher in cases at pneumonia diagnosis ($p=0.028$), while only controls showed a significant decrease over time ($p=0.007$) (figure 4b). SRSq and MS1 scores were strongly correlated (baseline $\rho=0.72$, event $\rho=0.73$; $p<0.0001$) (figure 4c and d).

Discussion

While immune dysregulation has been implicated in ICU patients' susceptibility to pneumonia, temporal changes in the host immune response prior to developing secondary infections are not well studied. To the best of our knowledge, our study is the first to characterise longitudinal changes in the blood transcriptome from baseline to the onset of ICU-acquired pneumonia. We used sequential data from two prospective cohorts to show that critically ill patients who develop pneumonia exhibit upregulated innate immunity pathways and downregulated adaptive immunity and cytokine signalling pathways at ICU admission, compared to controls. Progressing towards ICU-acquired pneumonia, cases demonstrated increased upregulation in innate immunity and haemostasis pathways, and downregulation of metabolic pathways, relative to controls. Moreover, cases showed increased SRSq and MS1 scores, indicators of immune dysregulation, at both baseline and pneumonia diagnosis. This study provides novel insights into the dynamic changes of the immune response in critically ill patients leading up to pneumonia, enhancing our understanding of pathophysiological mechanisms that may contribute to ICU-acquired infection development and potentially offering opportunities for predictive enrichment of immunomodulatory trials in this population.

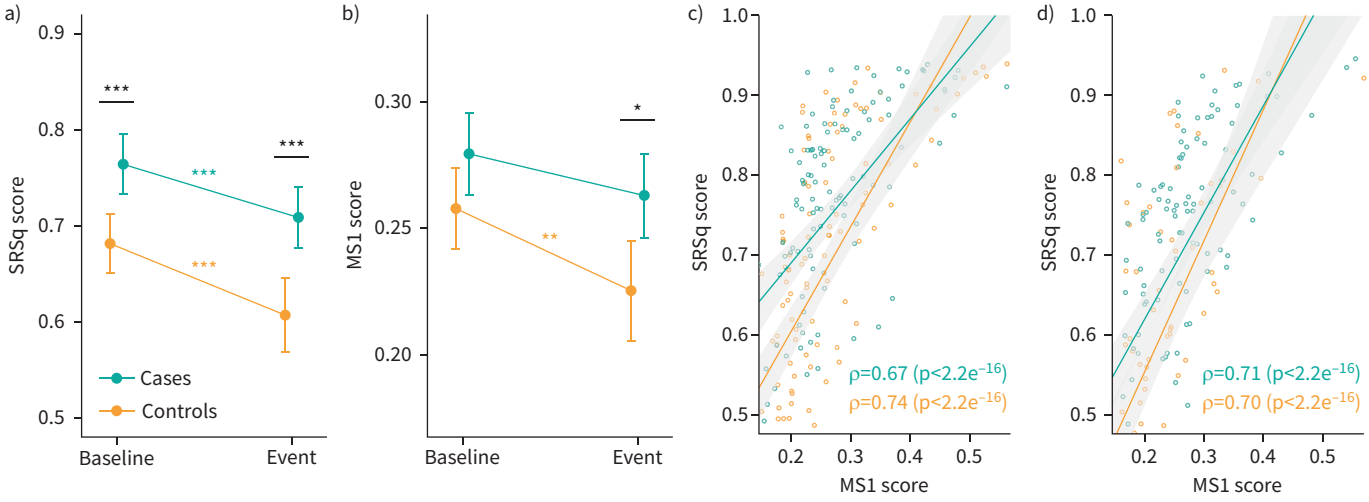


FIGURE 4 Quantitative sepsis response signature (SRSq) and monocyte state 1 (MS1) scores in critically ill trauma and surgery patients, stratified according to development of intensive care unit-acquired pneumonia or not. a) SRSq and b) MS1 scores in cases *versus* controls, at baseline, pneumonia event (in cases) or day 7 (in controls), and their change over time. Data are expressed as mean estimate with 95% confidence interval, derived from linear mixed models. Asterisks indicate significant differences between groups or over time. *: p<0.05; **: p<0.01; ***: p<0.001. c, d) Correlation between SRSq and MS1 score, calculated c) at baseline and d) at pneumonia event (in cases) *versus* day 7 (in controls). Values shown in the figure were derived from Spearman rank correlation analysis.

Previous research on ICU-acquired infections mainly focused on immunosuppressive features [5, 8, 26–32]. Our data highlight diverse immune alterations in patients developing ICU-acquired pneumonia, not limited to immunosuppression, evident at baseline and worsening towards pneumonia diagnosis. Accordingly, we recently reported broad plasma host response disturbances in ICU-acquired pneumonia, indicative of stronger pro-inflammatory, pro-coagulant and endothelial responses [12]. Activation of pattern recognition receptors, such as Toll-like receptors, by damage-associated molecular patterns may underlie these pro-inflammatory responses [6, 7, 10]. Similarly, we demonstrated aberrations across multiple pathophysiological domains in sepsis patients later developing ICU-acquired infections [11]. Correspondingly, two recent investigations reported enhanced innate immune gene expression in non-critically ill patients before post-operative infections [33, 34]. These data collectively suggest that ICU patients developing infections like pneumonia experience comprehensive immune dysregulation, extending beyond immunosuppression.

We evaluated two immune signatures: SRSq and MS1 scores [16, 17]. SRSq, indicative of immune dysfunction, is prognostic of outcomes such as mortality and secondary infections in sepsis [17]. A recent study reported higher SRSq scores in post-operative pneumonia patients compared to non-infected individuals [35]. We expand these data by showing elevated SRSq scores in trauma and surgery patients who develop ICU-acquired pneumonia, at both baseline and pneumonia diagnosis, compared to controls. Likewise, MS1 scores were higher in patients developing ICU-acquired pneumonia. SRSq and MS1 scores were strongly correlated, suggesting they capture similar immune distortions. Additionally, we identified two gene coexpression modules related to interferon/cytokine signalling and adaptive immunity, both downregulated in cases. Together, these findings suggest that at least part of the immune dysregulation in ICU-acquired pneumonia relates to immunosuppression.

This study has strengths and limitations. Patients were prospectively recruited across numerous European ICUs, increasing generalisability. Nonetheless, we specifically selected a relatively homogeneous patient group admitted for trauma or surgery. Participants were evaluated daily for ICU-acquired pneumonia by strict protocol definitions, universally endorsed by leading medical organisations [20, 36, 37]. Of note, this case definition includes both ventilator-associated and hospital-acquired pneumonia. Although respiratory specimens from all patients were cultured twice weekly, the exact onset of pneumonia and its microbiological aetiology are difficult to determine, and therefore transcriptomic patterns may not be entirely specific for pneumonia. Our findings were validated in an independent, longitudinally sampled cohort, strengthening result robustness; even so, the validation cohort comprised younger and more severely ill trauma patients compared with the derivation cohort. Although the overall results were largely comparable, small differences indicate that our results should be interpreted with caution and cannot

simply be generalised to other ICU populations, including those entailing non-trauma and non-surgical patients. The derivation cohort lacked detailed data on treatments. Inherent to the study design, some controls were either discharged or deceased prior to follow-up sampling, presenting competing risks that may influence results in opposite directions. To address potential bias from this informative dropout, we conducted additional analyses restricted to patients with ICU stays ⩾7 days, which corroborated our results; nonetheless, these sensitivity analyses may not have fully mitigated the impact of this dropout. Similarly, ICU patients are exposed to various "events" (*e.g.* complications or interventions) not causally linked to pneumonia, which may differ between cases and controls, and could influence blood transcriptome changes over time. Our cohort was enriched with *S. aureus*-colonised patients, potentially limiting generalisability; however, colonisation rates were comparable between cases and controls, and adjusting for colonisation status yielded similar results. Our analysis was limited to the systemic host response, without assessment of the local lung environment. While the transcriptome in nosocomial infections has been studied before, previous work has primarily been cross-sectional; our longitudinal approach offers new insights into the progression of the host response. However, the relative impact of immune suppression *versus* inflammation on pneumonia development could not be determined by this study design; causal relationships between host response changes and the occurrence of pneumonia cannot be established in observational studies. Thus, our results do not directly pinpoint treatable biological traits to prevent pneumonia, yet they do suggest that targeting one component of the aberrant immune response (*e.g.* the administration of a single immunostimulant) may have modest impact.

Conclusion

Immunostimulatory therapies have been explored for preventing nosocomial infections in the critically ill [5, 8]. A recent trial of the immunostimulatory cytokine interferon-γ-1b in mechanically ventilated patients was discontinued early because of potential harm and lack of effect on pneumonia and death rates [9]. We here describe increased upregulation of immune pathways in blood leukocytes of patients progressing towards ICU-acquiring pneumonia, particularly related to innate and adaptive immunity and haemostasis. While confirming immunosuppressive features in these patients, our data also suggest that measuring selective immune suppression readouts might overlook other significant immune anomalies, including pro-inflammatory reactions. These data are informative in the selection of ICU patients for immunostimulatory therapy. However, more data needs to be accumulated through further studies before advancing to therapeutic strategies.

Acknowledgements: The authors acknowledge all members of the ASPIRE-ICU study team (see supplementary material) for participation in sample and data collection, and the Host Response to Injury Investigators for their contribution. We also thank the National Institute of General Medical Sciences for their support of the Large-Scale Collaborative Project Award (U54GM062119).

Data availability: Gene expression data of the ASPIRE-ICU cohort are accessible under NCBI BioProject ID PRJNA1008068. Gene expression data from the validation cohort have been deposited in GEO DataSets under accession number GSE11375.

Ethics statement: This study was performed in line with the principles of the 1964 Declaration of Helsinki and its later amendments. The study protocol was approved by the institutional review boards or ethical committees in each country and/or site. All participants or their legally authorised representative provided written informed consent.

Author contributions: J. de Brabander, T.D.Y. Reijnders, T.S.R. van Engelen, F.P. Paling, M.J.M. Bonten, L. Timbermont, S. Malhotra-Kumar, J.A.J.W. Kluytmans, H. Peters-Sengers and T. van der Poll contributed to the conception and design of the study. F.P. Paling and L. Timbermont contributed to the acquisition of the samples and clinical data. T.D.Y. Reijnders, T.S.R. van Engelen, J.M. Butler, A.M. Klarenbeek, D.L.S. Sie and R.E. Boyer acquired the transcriptomic data. J. de Brabander, J.M. Butler and T. van der Poll had access to the raw data, performed the analyses and drafted the manuscript. E.H.A. Michels, T.D.Y. Reijnders, G.G.F. Leite, T.E. Sweeney and H. Peters-Sengers provided intellectual input and revised the initial draft. All authors and the collaborators approved the final version of the manuscript. T. van der Poll was responsible for the overall content as the guarantor.

Conflict of interest: The authors have no potential conflicts of interest to disclose.

Support statement: This paper was supported by Innovative Medicines Initiative Joint Undertakings 115523, 115620 and 115737 to M.J.M. Bonten. J. de Brabander and E.H.A. Michels were funded by the European Union's

Horizon 2020 research and innovation programme under grant number 847786 (FAIR). J.M. Butler was supported by the European Commission (Horizon 2020, ImmunoSep; grant number 847422). G.G.F. Leite was supported by FAPESP (grant 2022/06085–7). H. Peters-Sengers was supported by the Dutch Kidney Foundation (Kolff; grant number 19OK009). T.D.Y. Reijnders was supported by the Dutch Research Council (NWO) (NACTAR; grant number 16447). The funding sources had no role in the design of the study and collection, analysis, and interpretation of data. Funding information for this article has been deposited with the Crossref Funder Registry.

References

1 Kollef MH, Torres A, Shorr AF, *et al.* Nosocomial infection. *Crit Care Med* 2021; 49: 169–187.
2 Ferrer M, Torres A. Epidemiology of ICU-acquired pneumonia. *Curr Opin Crit Care* 2018; 24: 325–331.
3 Torres A, Niederman MS, Chastre J, *et al.* International ERS/ESICM/ESCMID/ALAT guidelines for the management of hospital-acquired pneumonia and ventilator-associated pneumonia. *Eur Respir J* 2017; 50: 1700582.
4 Paling FP, Hazard D, Bonten MJM, *et al.* Association of *Staphylococcus aureus* colonization and pneumonia in the intensive care unit. *JAMA Netw Open* 2020; 3: e2012741.
5 Venet F, Monneret G. Advances in the understanding and treatment of sepsis-induced immunosuppression. *Nat Rev Nephrol* 2018; 14: 121–137.
6 Lord JM, Midwinter MJ, Chen YF, *et al.* The systemic immune response to trauma: an overview of pathophysiology and treatment. *Lancet* 2014; 384: 1455–1465.
7 Hawkins RB, Raymond SL, Stortz JA, *et al.* Chronic critical illness and the persistent inflammation, immunosuppression, and catabolism syndrome. *Front Immunol* 2018; 9: 1511.
8 Torres LK, Pickkers P, van der Poll T. Sepsis-induced immunosuppression. *Annu Rev Physiol* 2022; 84: 157–181.
9 Roquilly A, Francois B, Huet O, *et al.* Interferon gamma-1b for the prevention of hospital-acquired pneumonia in critically ill patients: a phase 2, placebo-controlled randomized clinical trial. *Intensive Care Med* 2023; 49: 530–544.
10 van der Poll T, Shankar-Hari M, Wiersinga WJ. The immunology of sepsis. *Immunity* 2021; 54: 2450–2464.
11 van Vught LA, Wiewel MA, Hoogendijk AJ, *et al.* The host response in patients with sepsis developing intensive care unit-acquired secondary infections. *Am J Respir Crit Care Med* 2017; 196: 458–470.
12 van Engelen TSR, Reijnders TDY, Paling FP, *et al.* Plasma protein biomarkers reflective of the host response in patients developing intensive care unit-acquired pneumonia. *Crit Care* 2023; 27: 269.
13 Xiao W, Mindrinos MN, Seok J, *et al.* A genomic storm in critically injured humans. *J Exp Med* 2011; 208: 2581–2590.
14 Davenport EE, Burnham KL, Radhakrishnan J, *et al.* Genomic landscape of the individual host response and outcomes in sepsis: a prospective cohort study. *Lancet Respir Med* 2016; 4: 259–271.
15 Scicluna BP, van Vught LA, Zwinderman AH, *et al.* Classification of patients with sepsis according to blood genomic endotype: a prospective cohort study. *Lancet Respir Med* 2017; 5: 816–826.
16 Reyes M, Filbin MR, Bhattacharyya RP, *et al.* An immune-cell signature of bacterial sepsis. *Nat Med* 2020; 26: 333–340.
17 Cano-Gamez E, Burnham KL, Goh C, *et al.* An immune dysfunction score for stratification of patients with acute infection based on whole-blood gene expression. *Sci Transl Med* 2022; 14: eabq4433.
18 van Vught LA, Klein Klouwenberg PM, Spitoni C, *et al.* Incidence, risk factors, and attributable mortality of secondary infections in the intensive care unit after admission for sepsis. *JAMA* 2016; 315: 1469–1479.
19 Paling FP, Troeman DPR, Wolkewitz M, *et al.* Rationale and design of ASPIRE-ICU: a prospective cohort study on the incidence and predictors of *Staphylococcus aureus* and *Pseudomonas aeruginosa* pneumonia in the ICU. *BMC Infect Dis* 2017; 17: 643.
20 National Healthcare Safety Network. Patient Safety Component Manual. Atlanta, Centers for Disease Control and Prevention, 2023.
21 Pearce N. Analysis of matched case-control studies. *BMJ* 2016; 352: i969.
22 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; 15: 550.
23 Gillespie M, Jassal B, Stephan R, *et al.* The reactome pathway knowledgebase 2022. *Nucleic Acids Res* 2022; 50: D687–D692.
24 Russo PST, Ferreira GR, Cardozo LE, *et al.* CEMiTool: a Bioconductor package for performing comprehensive modular co-expression analyses. *BMC Bioinformatics* 2018; 19: 56.
25 Lewis M, Goldmann K, Sciacca E, *et al.* glmmSeq: general linear mixed models for gene-level differential expression. R package version 0.5.4. 2022. https://github.com/myles-lewis/glmmSeq Date last accessed: 10 January 2025.
26 Landelle C, Lepape A, Voirin N, *et al.* Low monocyte human leukocyte antigen-DR is independently associated with nosocomial infections after septic shock. *Intensive Care Med* 2010; 36: 1859–1866.

27 Wu JF, Ma J, Chen J, *et al.* Changes of monocyte human leukocyte antigen-DR expression as a reliable predictor of mortality in severe sepsis. *Crit Care* 2011; 15: R220.

28 Gouel-Cheron A, Allaouchiche B, Floccard B, *et al.* Early daily mHLA-DR monitoring predicts forthcoming sepsis in severe trauma patients. *Intensive Care Med* 2015; 41: 2229–2230.

29 Cajander S, Backman A, Tina E, *et al.* Preliminary results in quantitation of HLA-DRA by real-time PCR: a promising approach to identify immunosuppression in sepsis. *Crit Care* 2013; 17: R223.

30 Le Tulzo Y, Pangault C, Amiot L, *et al.* Monocyte human leukocyte antigen-DR transcriptional downregulation by cortisol during septic shock. *Am J Respir Crit Care Med* 2004; 169: 1144–1151.

31 Cheron A, Floccard B, Allaouchiche B, *et al.* Lack of recovery in monocyte human leukocyte antigen-DR expression is independently associated with the development of sepsis after major trauma. *Crit Care* 2010; 14: R208.

32 Lukaszewicz AC, Grienay M, Resche-Rigon M, *et al.* Monocytic HLA-DR expression in intensive care patients: interest for prognosis and secondary infection prediction. *Crit Care Med* 2009; 37: 2746–2752.

33 Namas RA, Vodovotz Y, Almahmoud K, *et al.* Temporal patterns of circulating inflammation biomarker networks differentiate susceptibility to nosocomial infection following blunt trauma in humans. *Ann Surg* 2016; 263: 191–198.

34 Lukaszewski RA, Jones HE, Gersuk VH, *et al.* Presymptomatic diagnosis of postoperative infection and sepsis using gene expression signatures. *Intensive Care Med* 2022; 48: 1133–1143.

35 Torrance HD, Zhang P, Longbottom ER, *et al.* A transcriptomic approach to understand patient susceptibility to pneumonia after abdominal surgery. *Ann Surg* 2024; 279: 510–520.

36 Committee for Human Medicinal Products. Addendum to the Guideline on the Evaluation of Medicinal Products Indicated for Treatment of Bacterial Infections. London, European Medicines Agency, 2013.

37 Center for Drug Evaluation and Research. Hospital-Acquired Bacterial Pneumonia and Ventilator-Associated Bacterial Pneumonia: Developing Drugs for Treatment Guidance for Industry. Silver Spring, Food and Drug Administration, 2020.