Blood leukocyte transcriptomes in Gram-positive and Gram-negative community-acquired pneumonia

Liza Pereverzeva[1], Fabrice Uhel[1], Hessel Peters Sengers[1], Joe Butler[1], Lonneke A. van Vught[1], Katie L. Burnham[2], Emma E. Davenport[2], Julian C. Knight[3], Olaf L. Cremer[4], Marcus J. Schultz[5,6,7], Marc M.J. Bonten[8,9], Brendon P. Scicluna[1,10] and Tom van der Poll[1,11] on behalf of the MARS consortium

[1]Center for Experimental and Molecular Medicine, Amsterdam University Medical Centers, location Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. [2]Wellcome Sanger Institute, Cambridge, UK. [3]Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK. [4]Dept of Intensive Care Medicine, University Medical Center Utrecht, Utrecht, The Netherlands. [5]Dept of Intensive Care, Amsterdam University Medical Centers, location Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. [6]Mahidol-Oxford Tropical Medicine Research Unit (MORU), Mahidol University, Bangkok, Thailand. [7]Nuffield Dept of Medicine, University of Oxford, Oxford, UK. [8]Dept of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands. [9]Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands. [10]Dept of Clinical Epidemiology, Biostatistics and Bioinformatics, Amsterdam University Medical Centers, location Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. [11]Division of Infectious Diseases, Amsterdam University Medical Centers, location Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Corresponding author: Liza Pereverzeva (e.pereverzeva@amsterdamumc.nl)

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Outcome and host response in critically ill patients with community-acquired pneumonia are similar when caused by Gram-positive compared with Gram-negative bacteria https://bit.ly/3rMll2r

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Abstract

Background Gram-positive and Gram-negative bacteria are the most common causative pathogens in community-acquired pneumonia (CAP) on the intensive care unit (ICU). The aim of this study was to determine whether the host immune response differs between Gram-positive and Gram-negative CAP upon ICU admission.

Methods 16 host response biomarkers providing insight into pathophysiological mechanisms implicated in sepsis and blood leukocyte transcriptomes were analysed in patients with CAP upon ICU admission in two tertiary hospitals in the Netherlands.

Results 309 patients with CAP with a definite or probable likelihood (determined by predefined criteria) were included. A causative pathogen was determined in 74.4% of admissions. Patients admitted with Gram-positive CAP (n=90) were not different from those admitted with Gram-negative CAP (n=75) regarding demographics, chronic comorbidities, severity of disease and mortality. Host response biomarkers reflective of systemic inflammation, coagulation activation and endothelial cell function, as well as blood leukocyte transcriptomes, were largely similar between Gram-positive and Gram-negative CAP. Blood leukocyte transcriptomes were also similar in Gram-positive and Gram-negative CAP in two independent validation cohorts. On a pathogen-specific level, *Streptococcus pneumoniae* and *Escherichia coli* induced the most distinct host immune response.

Conclusion Outcome and host response are similar in critically ill patients with CAP due to Gram-positive bacteria compared with Gram-negative bacteria.

Introduction

Community-acquired pneumonia (CAP) is the leading infectious cause of death worldwide, accounting for 3 million deaths annually [1]. In high-income countries, pneumonia is the most common cause of sepsis, responsible for roughly half of all sepsis cases [2]. Despite treatment with antimicrobial therapy, 10–20% of all adult patients hospitalised with CAP require supportive care at an intensive care unit (ICU) [3, 4].

The Gram-positive bacterium *Streptococcus pneumoniae* has been reported as the most common causative pathogen of CAP [5]. Recent studies show that the global aetiology of CAP is changing. In Asia, the

incidence of Gram-negative causative pathogens is increasing [6]. On the ICU, Gram-negative enteric bacteria and *Staphylococcus aureus* are more prevalent [7], and these pathogens, together with *Pseudomonas aeruginosa*, have been associated with higher mortality rates [8]. In spite of thorough microbiological testing on the ICU, the causative pathogen remains unknown in 45% of CAP cases in critically ill patients [7, 9].

Many investigations have reported on host response biomarkers in patients with CAP [10–13]. In general, these studies sought to determine the diagnostic and/or prognostic value of biomarkers with little attention to pathophysiological implications [10–13]. Numerous investigations aimed to determine the capacity of biomarkers such as C-reactive protein (CRP) and procalcitonin in discriminating between bacterial and viral respiratory tract infection. Only few studies compared the host response in CAP caused by different causative bacterial pathogens [14, 15]. These investigations only included CAP patients admitted to a general hospital ward (*i.e.* not an ICU), reported a limited number of biomarkers and primarily focused on plasma cytokine responses. Importantly, the host response during CAP requiring intensive care is likely to be different from that in CAP patients admitted to a general hospital ward, not only because of the different spectrum of causative microorganisms [7] but also considering the profoundly disturbed immunological and inflammatory homeostasis associated with critical illness *per se* [16, 17].

The aim of our study was to investigate whether the host immune response during CAP in critically ill patients varies between causative pathogens. We focused on differences between Gram-positive and Gram-negative causative bacteria, considering the more prominent role of enteric bacteria in CAP on the ICU [7, 18] and considering that lipopolysaccharide (one of the most potent biological products capable of activating immune cells) is uniquely expressed by Gram-negative bacteria [19, 20]. To this end we measured 16 biomarkers providing insight into deregulation of key host response pathways implicated in the pathogenesis of severe CAP (*i.e.* systemic inflammation, coagulation activation and endothelial dysfunction) and, in an unbiased way, analysed genome-wide transcriptomes in blood leukocytes.

Methods

Study design, setting and patient identification

This study was part of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) project, a prospective observational cohort study conducted in the ICUs of two hospitals in the Netherlands (Academic Medical Center in Amsterdam and University Medical Center Utrecht) (ClinicalTrials.gov: NCT01905033) [21, 22]. Between January 2011 and January 2014, all patients >18 years of age admitted with an expected length of stay >24 h were included *via* an opt-out method approved by the ethical committees of both participating hospitals.

For the current analysis, all consecutive patients suspected of CAP and for which the attending clinician initiated antibiotic treatment were included. Exclusion criteria were transfer to the ICU >48 h after admission to the ward, a history of respiratory symptoms >10 days before ICU admission, readmissions within the same hospital stay or within 30 days after discharge and transfers from another ICU, except when on the same day of presentation to the first ICU. For every patient, the likelihood of CAP was assessed by dedicated researchers making use of all clinical, radiological and microbiological data, and categorised on a four-point scale (ascending from none, possible, probable to definite) using criteria as described in detail in supplementary table S1 [21, 23]. For the final study population, admissions with the highest likelihood of CAP, *i.e.* probable or definite, were selected. For the comparison of Gram-positive with Gram-negative CAP, CAP caused by mixed Gram-positive/Gram-negative infection was excluded from analysis. For the comparison of the host response in CAP due to the five most common bacteria, only mono-infections were selected.

Clinical variables

Shock was defined by the use of vasopressors (norepinephrine, epinephrine or dopamine) in a norepinephrine-equivalent dose of >0.1 $\mu g \cdot kg^{-1} \cdot min^{-1}$. Comorbidities were defined as specified in the supplementary material. Acute kidney injury and acute respiratory distress syndrome (ARDS) were defined using strict pre-set criteria [24, 25]. ICU-acquired complications were defined when they occurred >48 h after ICU admission.

Plasma biomarker measurements

Measurements were done in EDTA anticoagulated plasma collected on ICU admission as described in the supplementary material.

Blood gene expression microarrays

Blood leukocyte gene profiles were determined in patients enrolled during the first 1.5 years of the study. Whole blood was drawn in PAXgene tubes (Becton Dickinson, Breda, The Netherlands) within 24 h after ICU admission and from 42 healthy controls (median (interquartile range (IQR)) age 35 (30–63) years; 57% male) after having obtained written informed consent. Total RNA was isolated, processed and hybridised to the Affymetrix Human Genome U219 96-array plate (Thermo Fisher Scientific, Waltham, MA, USA) and analysed as described in the supplementary material.

Validation cohorts

Blood leukocyte transcriptome data were validated in two independent cohorts [26, 27]. For details, see the supplementary material.

Statistical analysis

Continuous variables are presented as mean with standard deviation and were compared using the t-test or ANOVA when normally distributed, and presented as median (IQR) and analysed using the parametric Mann–Whitney U-test or Kruskal–Wallis test when not. Categorical variables are presented as numbers (percentages) and were analysed using the Chi-squared test or Fisher's exact test. For multiple group comparisons, *post hoc* testing was done using Dunn's test of multiple comparisons using rank sums (continuous variables) or a pairwise test for a multilevel two-dimensional matrix (categorical variables). Kaplan–Meier curves were used to plot 30-day survival. For the plasma biomarker outcomes of specific causative pathogens, linear regression was used with contrast dummy coding for causative pathogen categories. To meet the normality assumption, $\log_{10}$ or Box–Cox transformation was used. Overall group difference was tested by Wald Chi-squared statistics, adjusted for multiple testing with the Benjamini–Hochberg (BH) false discovery rate approach. If the overall group difference was deemed significant, each category of the causative pathogen was compared with the reference category to identify particular differences between pairs. Calculation of principal component analysis plots was done by a singular value decomposition of the centred and scaled data matrix including gene expression.

All analyses were performed in R studio version 4.0.2 (www.r-project.org) with the survival (3.1–8), ggplot2 (3.3.0), prcomp, pheatmap (1.0) and limma (3.46) packages. Nominal and multiple-comparison-adjusted p-values<0.05 were considered to be of statistical significance. For differential gene expression and pathway enrichment, BH-adjusted p-values <0.05 defined significance.

Results

Patients, microbiology and outcome

The 3-year study period entailed 954 ICU admissions with suspected CAP (figure 1). Of these, 239 admissions (25.1%) were excluded because of a preceding stay on the ward for ⩾48 h (n=186), respiratory symptoms for >10 days before ICU admission (n=63), readmission within the same hospital stay or within 30 days after discharge (n=50), or transfer from another ICU (n=45). Multiple exclusion criteria were met in 79 admissions. Of the 715 remaining admissions, the likelihood of CAP was classified as definite in 96 (13.4%) cases, probable in 213 (30.0%), possible in 271 (37.9%) and the diagnosis was refuted (likelihood none) in 135 (18.9%). The final cohort consisted of 213 admissions for probable CAP and 96 admissions for definite CAP.

A causative pathogen could be identified in 74.4% of these admissions (supplementary figure S1 and supplementary table S2). In total, 358 pathogens were isolated; more than one causative pathogen was found in 13.6% of cases, and these were mostly co-infections of Gram-positive and Gram-negative bacteria (n=16) or bacteria and viruses (n=13). Gram-positive bacteria were cultured in 36.6% of cases and Gram-negative bacteria were cultured in 31.1% of cases (supplementary table S2). *S. pneumoniae* was the most common causative pathogen (18.4%), followed by *S. aureus* (9.8%) and *Haemophilus influenzae* (7.5%).

Patients admitted with Gram-positive bacterial CAP and those admitted with Gram-negative bacterial CAP were not different in terms of demographics and chronic comorbidities (table 1). Patients with Gram-negative CAP were more often admitted from an assisted living facility (p=0.010). The severity of disease on ICU admission was comparable between groups, as indicated by similar Acute Physiology and Chronic Health Evaluation IV and Sequential Organ Failure Assessment scores, as well as similar percentages of mechanical ventilation requirement. Patients with Gram-positive CAP tended to have shock more often (64.4% *versus* 48.0%; p=0.049). Outcomes including 30-day mortality were not different between groups (table 1 and supplementary figure S2).

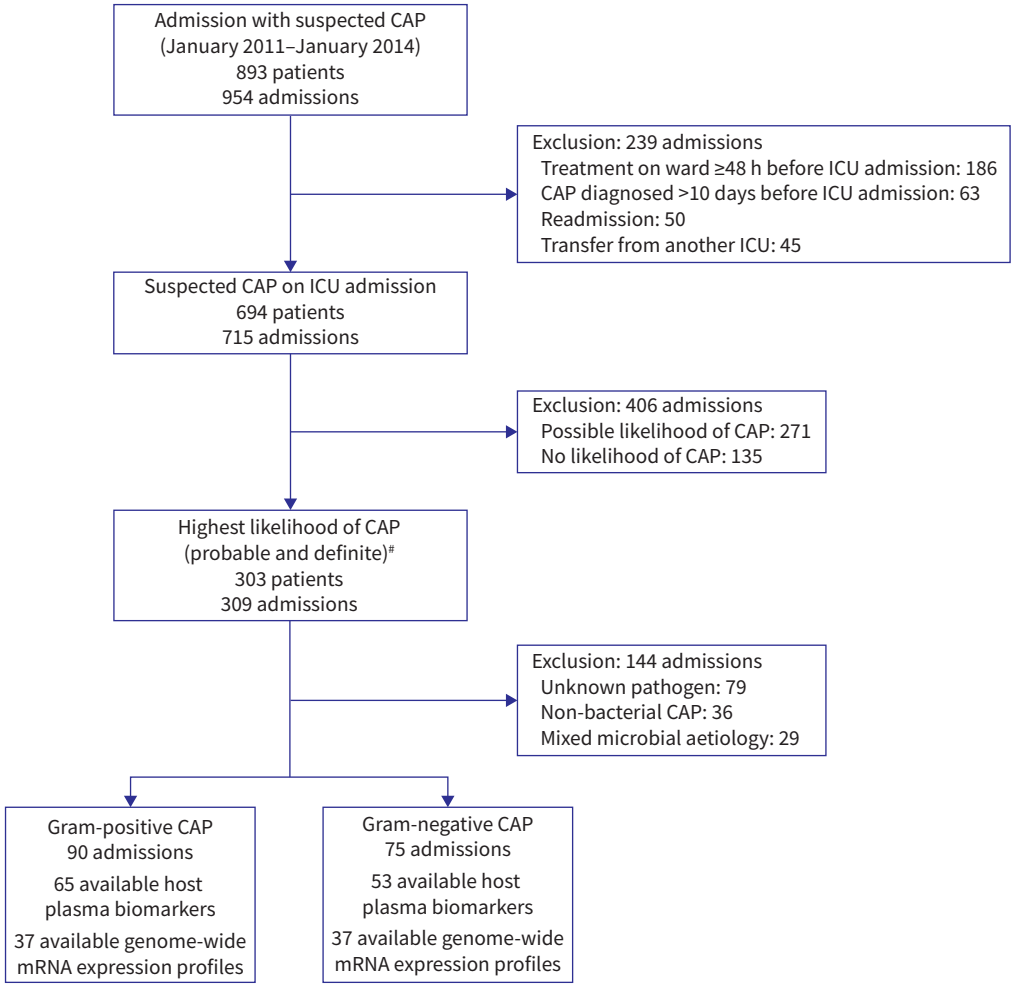

FIGURE 1 Flowchart of included patients and their likelihood of community-acquired pneumonia (CAP). Multiple exclusion criteria were met in 79 admissions. #: supplementary figure S1 and supplementary table S2. ICU: intensive care unit.

Plasma host response biomarkers

Plasma biomarkers reflecting aberrations in key pathways implicated in sepsis pathogenesis were measured on ICU admission in 65 patients with Gram-positive and 53 patients with Gram-negative bacterial CAP (figure 2 and supplementary table S3). Compared with healthy controls, patients with either Gram-positive or Gram-negative bacterial CAP showed enhanced systemic inflammatory and cytokine responses (illustrated by elevated levels of CRP, interleukin (IL)-6, IL-8, IL-10 and matrix metalloproteinase (MMP)-8), activation of the coagulation system (elevated D-dimer, prolonged prothrombin time, prolonged activated partial thromboplastin time, and reduced protein C and antithrombin levels) and endothelial cell activation and dysfunction (increased soluble E-selectin, soluble intercellular adhesion molecule-1, fractalkine and angiopoietin-2/angiopoietin-1 ratio). These responses were similar between patients with Gram-positive or Gram-negative bacterial CAP, with the exception of MMP-8 levels that were significantly higher in Gram-positive bacterial CAP ($p<0.001$). The plasma levels of tumour necrosis factor (TNF)-α, IL-1β, IL-13 and interferon-γ were low or not detectable, and not different between groups (data not shown).

To obtain insight into the pathogen-specific host response in CAP, we further analysed CAP caused by one of the five most common bacterial pathogens: *S. pneumoniae* (n=49), *S. aureus* (n=22), *H. influenzae* (n=17), *P. aeruginosa* (n=15) and *Escherichia coli* (n=13). While demographics, chronic comorbidities and severity of disease on ICU admission were comparable between patients with CAP caused by these five pathogens, patients with CAP due to *E. coli* showed an increased early mortality (ICU mortality

TABLE 1 Baseline characteristics and outcome of patients admitted to the intensive care unit with Gram-positive or Gram-negative bacterial community-acquired pneumonia (CAP)

	Gram-positive bacterial CAP	Gram-negative bacterial CAP	p-value
Admissions	90	75	
Demographics			
Age (years)	62 (47–72)	64 (52–74)	0.37
Gender: male	58 (64.4)	47 (62.7)	0.94
Body mass index (kg·m^{-2})	24 (21–26)	24 (22–27)	0.42
Race: White	77 (85.6)	65 (86.7)	>0.99
Medical admission	83 (92.2)	70 (93.3)	>0.99
Time between hospital presentation and ICU admission (h)	15 (10–21)	16 (12–22)	0.50
Readmission[#]	2 (2.2)	2 (2.7)	>0.99
Assisted living facility	0 (0.0)	7 (9.3)	0.010
Chronic comorbidity			
None	25 (27.8)	26 (34.7)	0.43
Cardiovascular disease	31 (34.4)	15 (20.0)	0.06
COPD	25 (27.8)	16 (21.3)	0.44
Diabetes	12 (13.3)	10 (13.3)	>0.99
Immunocompromised state	21 (23.3)	16 (21.3)	0.91
Liver cirrhosis	2 (2.2)	0 (0.0)	0.56
Malignancy	17 (18.9)	11 (14.7)	0.61
Renal insufficiency	9 (10.0)	11 (14.7)	0.50
Respiratory insufficiency	29 (32.2)	20 (26.7)	0.54
Charlson Comorbidity Index	4 (2–6)	4 (2–5)	0.64
Severity of disease on ICU admission			
APACHE IV score	82 (64–108)	78 (62–98)	0.51
SOFA total	7 (6–9)	7 (4–9)	0.09
Mechanical ventilation	76 (84.4)	65 (86.7)	0.86
Shock	58 (64.4)	36 (48.0)	0.049
Organ failure	87 (96.7)	72 (96.0)	>0.99
Acute kidney injury	38 (42.2)	23 (30.7)	0.17
Acute respiratory distress syndrome	23 (25.6)	27 (36.0)	0.20
Acute myocardial infarction	1 (1.1)	1 (1.3)	>0.99
Outcome			
Length of ICU stay (days)	5 (3–9)	5 (3–9)	0.97
Length of hospital stay (days)	14 (7–24)	14 (6–28)	0.86
ICU-acquired complications			
None	77 (85.6)	61 (81.3)	0.60
Acute kidney injury	8 (8.9)	7 (9.3)	>0.99
Acute respiratory distress syndrome	6 (6.7)	3 (4.0)	0.68
Mortality[¶]			
ICU	13 (14.8)	15 (20.5)	0.45
Hospital	20 (22.7)	22 (30.1)	0.38
30 days	21 (23.9)	22 (30.1)	0.47
60 days	25 (28.4)	28 (38.4)	0.24
90 days	29 (33.0)	31 (42.5)	0.28
1 year	35 (39.8)	36 (49.3)	0.29

Data are presented as n, median (interquartile range) or n (%), unless otherwise stated. COPD: chronic obstructive pulmonary disease; ICU: intensive care unit; APACHE: Acute Physiology and Chronic Health Evaluation; SOFA: Sequential Organ Failure Assessment. [#]: readmissions >30 days after hospital discharge; [¶]: mortality was calculated using the first ICU admission for each patient (readmissions were not included in this analysis).

46.2% *versus* up to 18.2% for other pathogens; overall p=0.039) (supplementary table S4) and highest 30-day mortality (supplementary figure S3). Comparison of host response biomarkers between CAP cases caused by these specific pathogens showed differences between groups with regard to IL-8, IL-10, MMP-8, soluble E-selectin, angiopoietin-2 and angiopoietin-2/angiopoietin-1 ratio (figure 3). These differences were driven by *S. pneumoniae* and *E. coli*.

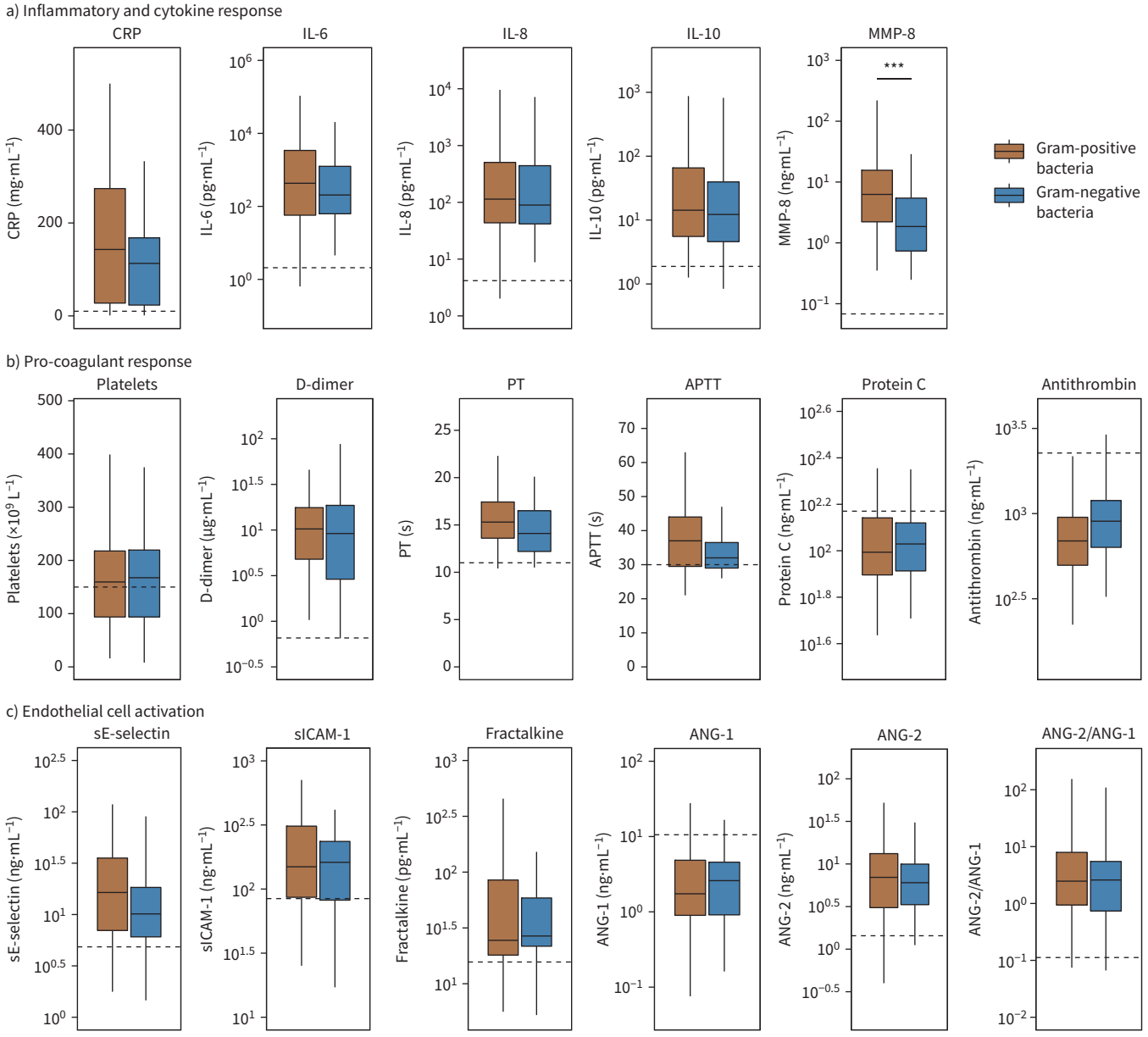

FIGURE 2 Host response plasma biomarkers in critically ill patients with Gram-positive or Gram-negative bacterial community-acquired pneumonia (CAP). Plasma biomarkers were measured on intensive care unit admission. Parameters are classified as a) inflammatory and cytokine response, b) coagulation activation, and c) endothelial cell activation biomarkers. Box-and-whisker plots depict median and interquartile range (IQR) with whiskers extending to 1.5 times the IQR of the lowest and highest quartile. Dotted lines indicate median values obtained in 27 healthy subjects. Values in patients were all significantly different from those in healthy controls, except for platelet counts. Differences between patients with CAP caused by Gram-positive bacteria compared with Gram-negative bacteria were analysed using the Mann–Whitney U-test, adjusted for multiple testing with the Benjamini–Hochberg false discovery rate approach. ***: p<0.001. CRP: C-reactive protein; IL: interleukin; MMP: matrix metalloproteinase; PT: prothrombin time; APTT: activated partial thromboplastin time; sICAM: soluble intercellular adhesion molecule; ANG: angiopoietin.

Blood leukocyte transcriptome analysis

Blood leukocyte genome-wide RNA profiles were determined on ICU admission in the subgroup of patients enrolled during the first 1.5 years of the study period (n=74, of whom 37 with Gram-positive CAP and 37 with Gram-negative CAP) (supplementary table S5). Principal component analysis revealed a large overlap in overall gene expression between Gram-positive and Gram-negative CAP (supplementary figure S4). Relative to healthy controls (n=42), patients with either Gram-positive or Gram-negative CAP displayed strong blood transcriptome alterations, encompassing 66–69% of all genes present on the array (figure 4a).

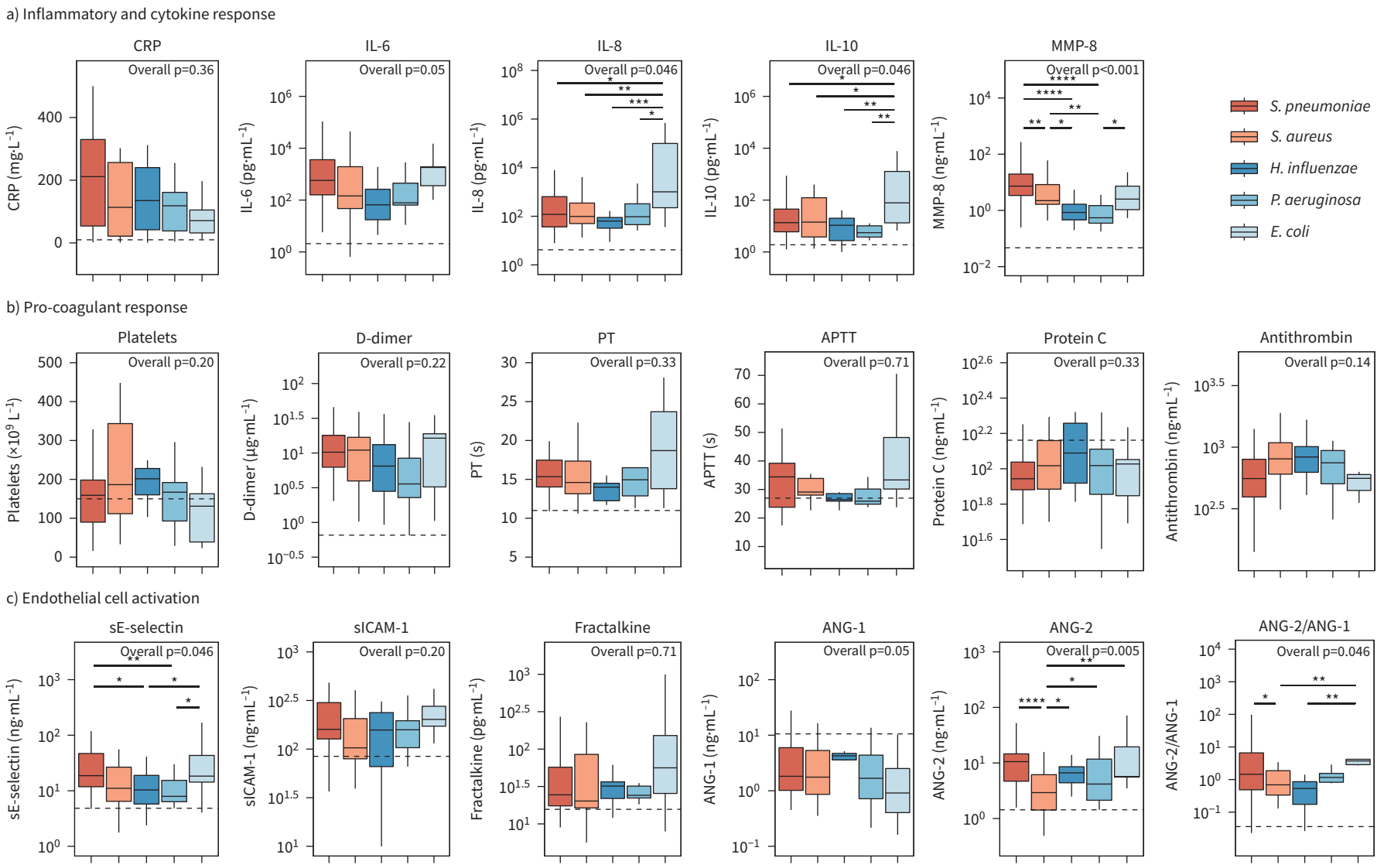

FIGURE 3 Host response plasma biomarkers in critically ill patients with community-acquired pneumonia (CAP) caused by one of five of the most common bacterial causative pathogens. Plasma biomarkers were measured on intensive care unit admission in patients with CAP due to *Streptococcus pneumoniae* (n=33), *Staphylococcus aureus* (n=18), *Haemophilus influenzae* (n=12), *Pseudomonas aeruginosa* (n=10) and *Escherichia coli* (n=9). Parameters are classified as a) inflammatory and cytokine response, b) coagulation activation, and c) endothelial cell activation biomarkers. Box-and-whisker plots depict median and interquartile range (IQR) with whiskers extending to 1.5 times the IQR of the lowest and highest quartile. Dotted lines indicate median values obtained in 27 healthy subjects. Overall group difference was tested by Wald Chi-squared statistics, adjusted for multiple testing with the Benjamini–Hochberg false discovery rate approach. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 using linear regression with contrast dummy coding for causative pathogen categories. CRP: C-reactive protein; IL: interleukin; MMP: matrix metalloproteinase; PT: prothrombin time; APTT: activated partial thromboplastin time; sICAM: soluble intercellular adhesion molecule; ANG: angiopoietin.

Of the altered transcriptomes, 79% were common to patients with CAP due to Gram-negative or Gram-positive bacteria (figure 4a) and this common transcriptional response showed strongly correlated gene expression fold changes (supplementary figure S5). In agreement, the top 10 most differentially regulated genes largely overlapped between Gram-positive and Gram-negative CAP (supplementary table S6). Consistent with earlier studies in CAP patients [28], pathway analysis of the common response

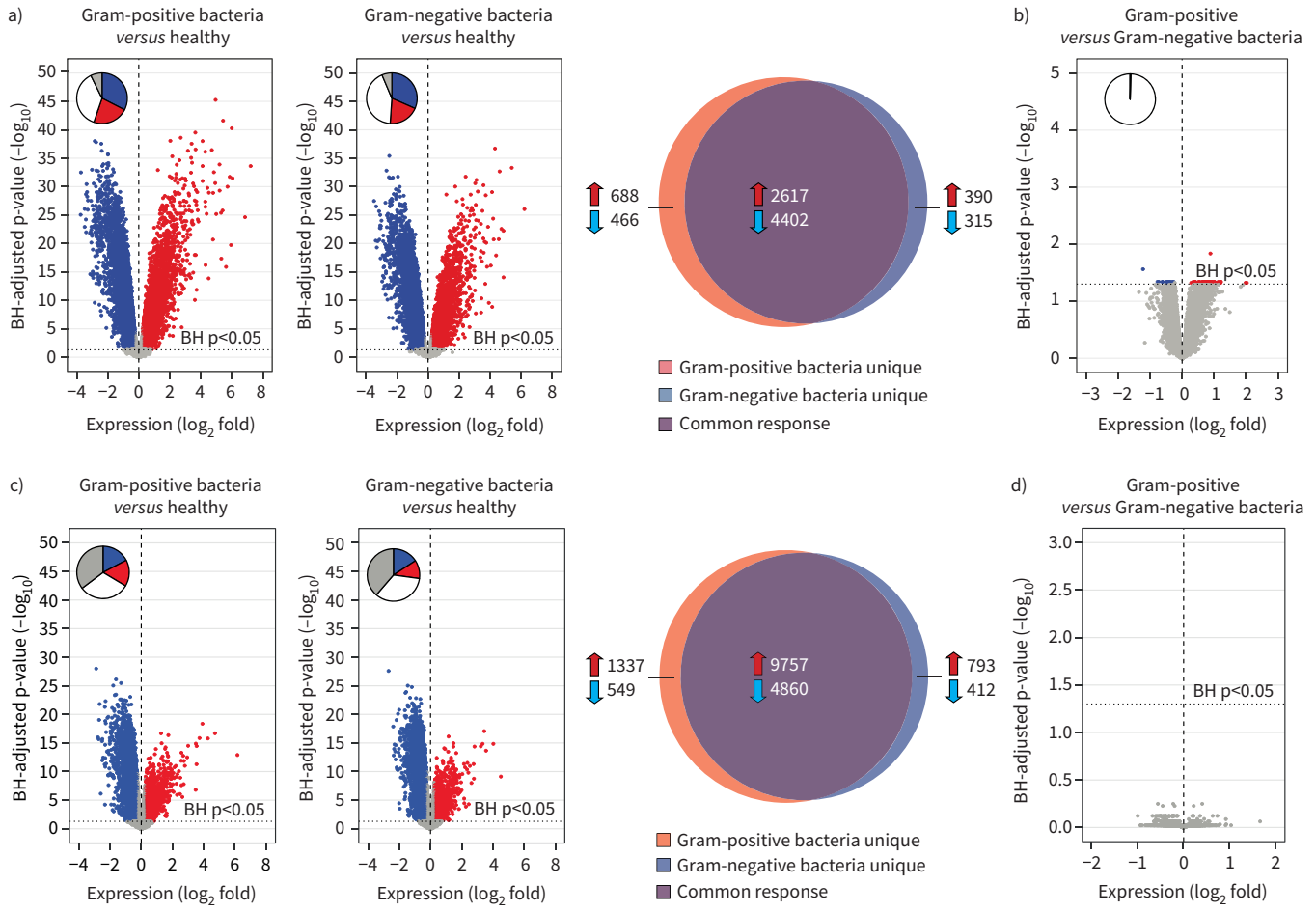

FIGURE 4 Leukocyte genomic responses upon admission in patients with Gram-positive or Gram-negative bacterial community-acquired pneumonia (CAP). a, c) Volcano plots illustrating the differences in leukocyte genomic responses (integrating $\log_2$ fold changes and multiple-test adjusted probabilities) between patients with CAP due to Gram-positive bacteria (left plot) or Gram-negative bacteria (right plot) and healthy subjects in the a) original and c) validation cohort. Blue dots represent significantly underexpressed genes (adjusted p<0.05, fold expression <−1.2) and red dots represent significantly overexpressed genes (adjusted p<0.05, fold expression >1.2) in patients relative to healthy controls. Horizontal dotted lines indicate the multiple-test adjusted Benjamini–Hochberg (BH) p<0.05 threshold. Inset pie charts show the extent of gene expression changes: blue slices show significantly underexpressed genes (adjusted p<0.05 and expression >1.2 times decreased compared with healthy controls), red slices show significantly overexpressed genes (adjusted p<0.05 and expression >1.2 times increased compared with healthy controls) and grey slices show significantly different gene expression (adjusted p<0.05 and expression <1.2 times increased or decreased compared with healthy controls). Venn–Euler diagrams represent differentially expressed genes on admission in patients with CAP due to Gram-positive or Gram-negative bacteria *versus* healthy subjects (adjusted p<0.05). Red arrows denote overexpressed genes and blue arrows denote underexpressed genes. b, d) Volcano plots illustrating the differences in leukocyte genomic responses on admission between patients with CAP due to Gram-positive bacteria and patients with CAP due to Gram-negative bacteria in the b) original and d) validation cohort. Data are presented as in a) and c). Inset pie charts show the extent of gene expression changes in Gram-positive compared with Gram-negative pneumonia. a) Considering adjusted p<0.05, 8173 and 7724 genes were identified as differentially expressed in patients with CAP due to Gram-positive or Gram-negative bacteria *versus* healthy subjects, respectively. b) Considering adjusted p<0.05, 74 genes were differentially expressed. Within plots, pie charts show the extent of gene expression changes in Gram-positive compared with Gram-negative pneumonia. c) Considering adjusted p<0.05, 16 503 and 15 822 genes were identified as differentially expressed in patients with CAP due to Gram-positive or Gram-negative bacteria *versus* healthy subjects, respectively. d) Considering adjusted p<0.05, no genes were differentially expressed. BH-adjusted p-value ($-\log_{10}$): negative $\log_{10}$-transformed p-value corrected for multiple comparisons.

revealed a typical overexpression of genes involved in both pro-inflammatory (IL-1, IL-8, inflammasome, TREM-1 signalling) and anti-inflammatory (IL-10 signalling) innate immune responses and metabolic pathways (mitochondrial dysfunction, hypoxia-inducible factor-1α signalling), and a concomitant underexpression of genes of lymphocyte (B-cell development, T-helper Th1 and Th2 activation, T-cell receptor signalling pathways), antigen presentation and mechanistic target of rapamycin pathways (supplementary figure S6). Genes involved in those pathways are depicted in supplementary figure S7. Differential gene expression analysis of patients with CAP due to Gram-positive relative to Gram-negative bacteria revealed limited differences between groups, encompassing 74 significantly altered genes (multiple-comparison-adjusted $p<0.05$) (figure 4b). Pathway analysis showed that underexpressed genes in patients with CAP due to Gram-positive compared with Gram-negative bacteria were significantly associated with a more severe impairment of pathways linked to adaptive immunity, especially pathways involving lymphocytes (supplementary figure S8). Overexpressed genes were not significantly associated to specific pathways.

Comparison of blood leukocyte transcriptomes of patients with CAP caused by the five most common causative pathogens (Gram-positive: *S. pneumoniae* and *S. aureus*; Gram-negative: *H. influenzae*, *P. aeruginosa* and *E. coli*) revealed marked differences with leukocyte transcriptomes from healthy controls (supplementary figures S9 and S10, and supplementary table S7). Direct comparison between causative pathogens within the Gram-negative group also disclosed some differences in blood leukocyte transcriptomes (supplementary figure S10d and supplementary table S8), but not within the Gram-positive group (supplementary figure S10c).

Validation of blood leukocyte transcriptome data

Validation of gene expression profiles in blood leukocytes from critically ill CAP patients caused by Gram-positive or Gram-negative pathogens was done in two independent cohorts (supplementary tables S9 and S10) [26, 27]. These data confirmed a large overlap in gene expression between Gram-positive and Gram-negative CAP compared with healthy controls (figure 4c). Moreover, pathway analysis of the common response revealed underexpression of genes of lymphocyte and metabolic pathways (supplementary figure S11). However, direct comparison between the two groups did not reveal significantly altered genes (figure 4d). The absence of significant differential gene expression of patients with CAP due to Gram-positive (n=60) relative to Gram-negative bacteria (n=35) was also found in the second validation cohort (the GAinS cohort; supplementary figure S12). Comparing leukocyte transcriptomes between individual pathogens was not possible in the validation cohorts due to the low sample size.

Discussion

The aim of the present study was to determine whether differences exist between the host response elicited by Gram-positive *versus* Gram-negative causative organisms in patients with CAP requiring intensive care. By measuring 16 biomarkers in the circulation and by analysing genome-wide mRNA expression profiles in blood leukocytes we show that the host response in Gram-positive and Gram-negative CAP is largely similar on admission to the ICU. Likewise, clinical presentation and mortality were comparable in patients with Gram-positive or Gram-negative CAP. On a pathogen-specific level, *S. pneumoniae* and *E. coli* induced the most distinct host immune response.

While most previous studies conducted in the ICU reported an unknown aetiology in ∼45% of CAP patients [7], a causative pathogen was determined in 74% of our patients. This could be explained by our strict diagnostic criteria for CAP, applied by a dedicated team of researchers that scored the likelihood of CAP making use of all relevant data collected after admission [21, 23]. This led to refutation of the diagnosis in 18.9% of suspected CAP and the exclusion of another 271 cases in which the likelihood of CAP was only scored as possible. Similar to other studies [7, 8, 29, 30], *S. pneumoniae* was the most common causative pathogen in our ICU cohort and the proportion of other causative pathogens also was akin to earlier surveys on the ICU, making the results generalisable to other populations. Only 24 patients (7.8%) were diagnosed with viral infection and 13 patients (4.2%) were diagnosed with mixed viral/bacterial CAP. Of note, this study was purely observational, reflecting common clinical practice, in which only 40% of patients were tested for respiratory viruses, suggesting that viruses might have been underreported [31]. Indeed, studies in which both bacterial and viral testing were performed systematically reported that respiratory viruses are isolated at least as often as bacteria from pneumonia patients on the ICU [3, 32, 33].

Patients with CAP caused by Gram-positive bacteria presented with similar comorbidities, disease severity and mortality rates compared with patients with Gram-negative CAP. Moreover, the evolution was similar

between Gram-positive and Gram-negative bacterial CAP, with comparable lengths of hospital stay and incidence of ICU-acquired complications. We found no significant difference in the incidence of ARDS between patients with CAP due to different pathogens, neither on admission nor acquired during ICU stay. In accordance, ARDS was reported to occur in 29% of mechanically ventilated CAP patients, independent of its aetiology [29]. Previous investigations on biomarkers in CAP have mostly been performed in the emergency room or general hospital ward and have focused on their value in discriminating bacterial from viral disease and prognosis [10–13]. Our study is different in that we did not seek to evaluate biomarkers as potential diagnostic or prognostic tools, but rather to obtain insight into differences between pathophysiological mechanisms at play during Gram-negative and Gram-positive CAP upon admission to the ICU, *i.e.* in the context of critical illness. In a targeted approach we focused on biomarkers reflecting aberrations in host response pathways considered to be involved in the pathogenesis of sepsis [34, 35]. As reported previously [10, 23, 36–38], CAP patients, relative to healthy subjects, presented with signs of systemic inflammation, activation of coagulation and endothelial cell dysfunction irrespective of the type of causative microorganism. Most of these responses were not different between Gram-positive and Gram-negative CAP with the exception of increased MMP-8 levels in the former group, along with a trend in higher soluble E-selectin, which was significant prior to adjustment for multiple testing. Of interest, in a previous investigation from our group, critically ill patients with CAP were reported to have higher MMP-8 and soluble E-selectin levels compared with patients with hospital-acquired pneumonia (HAP) [23]. Considering that the proportion of Gram-positive infection was much higher in CAP than in HAP patients [23], this previous study also hints at stronger induction of MMP-8 and endothelial cell activation by Gram-positive bacteria than by Gram-negative microorganisms during pneumonia. A study with hospitalised CAP patients reported similar CRP, TNF and IL-6 levels in patients with documented Gram-positive or Gram-negative infection; only IL-8 differed between groups, with higher levels in patients with Gram-negative CAP, which was driven by infections caused by Enterobacteriaceae [14]. In our cohort, we observed that *S. pneumoniae* and *E. coli* elicited the strongest systemic responses.

To analyse the host response between Gram-positive and Gram-negative CAP in the ICU in an unbiased way, we assessed the genome-wide transcriptomes in blood leukocytes. Previous studies have documented that blood leukocyte gene expression profiles are strongly altered in critically ill patients admitted to the ICU, but largely similar between different conditions such as Gram-positive and Gram-negative infections in general, community- and hospital-acquired infections, and even sepsis and trauma [23, 39, 40]. In agreement, in our cohort, gene expression in Gram-positive and Gram-negative bacterial CAP was largely common, characterised by an upregulated innate immune response, as well as downregulated genes related to adaptive immunity. However, we found Gram-positive bacteria to be associated with a more severe impairment of lymphocyte and IL-3 signalling. Interestingly, a study analysing plasma biomarkers to distinguish bloodstream infections caused by different species found IL-3 levels to be increased in patients with Gram-positive infection compared with Gram-negative infection [41]. Of note, however, we did not confirm these differences in leukocyte transcriptomes of patients with Gram-positive *versus* Gram-negative CAP in two independent validation cohorts. These validations did confirm the similar gene expression pattern in blood leukocytes from patients with Gram-positive and Gram-negative bacterial CAP.

Our study has strengths and limitations. This is the first study to investigate the influence of the causative pathogen on the host immune response in ICU patients with CAP, by using a large set of host response plasma biomarkers and whole-genome blood leukocyte transcriptome analysis. Moreover, this is the first study to compare clinical characteristics and outcome based on the causative pathogen of CAP in patients admitted to the ICU. Our investigation was purely observational and therefore does not establish a causal relationship between causative pathogen and host response. Furthermore, data on the blood leukocyte transcriptomes in CAP caused by individual microorganisms should be considered with caution due to low sample sizes.

In conclusion, critically ill patients with CAP caused by Gram-positive bacteria had similar outcomes and a largely overlapping host response compared with CAP caused by Gram-negative bacteria.

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